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THE EFFECT OF PAINTING THE PANCREAS WITH ADRENALIN UPON HYPERGLYCEMIA AND GLYCOSURIA.

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INTRODUCTION.

About a year after the discovery by Blum[1] that subcutaneous injections of adrenal extracts produce glycosuria, Herter and his coworkers[2] published several communications on the sugar-producing effect of adrenal substance when administered intraperitoneally. They employed adrenalin chloride (Takamine, 1: 1,000). According to Herter, intraperitoneal injections of adrenalin give better results than subcutaneous or intravenous injections. Herter's communications contain further the following striking statements: that painting of the pancreas with adrenalin brings on glycosuria which amounts often to 10 per cent and more, and that such a remarkable production of glycosuria may be brought about even by painting only one-fifth of the pancreas with adrenalin. He further states that "the pronounced nature of the glycosuria following intraperitoneal injections appears to be mainly attributable to the readiness with which the adrenalin comes into contact with the pancreas." The glycosuric action of painting the pancreas is brought into connection with the fact that glycosuria invariably makes its appearance after complete depancreatization, and the theory is advanced that the painting of the pancreas with adrenalin acts upon the cells of this organ in the manner of the action of hydrocyanic acid; namely, by the prevention of oxygenation of the glandular cells. In the discussion following this communication at the meeting of the Association of American Physicians, Herter stated that he was unable to offer a satisfactory reply to the question of one of us; namely, why the prevention of oxygenation of the cells of one-fifth of the pancreas should produce glycosuria, while the removal of even more than one-half of that gland produces no glycosuria. To the remarks of Cushing that the operative procedure,

[1] Blum, F., *Deutsch. Arch. klin. Med.*, 1901, lxxi, 146.

[2] Herter, C. A., and Richards, A. N., *Med. News*, 1902, lxxx, 201. Herter, C. A., *ibid.*, 1902, lxxx, 865. Herter, C. A., and Wakeman, A. J., *Tr. Assn. Am. Phys.*, 1902, xvii, 570; *Virchows Arch. path. Anat.*, 1902, clxix, 479. Vosburgh, C. H., and Richards, A. N., *Am. J. Physiol.*, 1903, ix, 35.

etc., might have been responsible for the glycosuria, Herter said that he "used only ether, and after you have used this for hours on dogs and examined the urine you get only a trace of sugar or none whatever." He admits that in a few instances the amount of glycosuria is inconsiderable and in exceptional instances glycosuria may be entirely absent. However, Herter's papers do not contain figures recording the number of successful experiments after painting the pancreas or after intraperitoneal injections, or indicating the degrees of the results.

In Herter and Wakeman's communications[2] there is only an unimportant reference to the hyperglycemic effect of painting. But later Vosburgh and Richards[2] report eight experiments in which the hyperglycemia was studied, five after painting the pancreas and three after intraperitoneal injections of adrenalin chloride. In these instances the increase in hyperglycemia was, indeed, considerable; but no statement is made as to the glycosuric effects. Vosburgh and Richards acknowledge the influence of ether upon the sugar content of the blood and the necessity of having controls for the experiments; they consider as a control the first analysis for blood sugar which has been made after anesthesia has been established and before adrenalin is used. In two of these controls the glycemia was high (0.239 and 0.258 per cent).

These statements are striking. Although more than 14 years have passed the experiments seem not to have been confirmed by other workers. We report below the results which were obtained in the repetition of some of the experiments.

EXPERIMENTAL.

Method.—The experiments were made on dogs. They were anesthetized with ether, some by the intratracheal insufflation method, some received ether by cone or towel, and in others ether was insufflated through a cone. In all cases urine and blood were analyzed for sugar content, the blood was analyzed once before painting the pancreas with adrenalin (Parke, Davis and Company, 1: 1,000), and several times at intervals after painting. Blood was obtained from a cannula in an artery (carotid or femoral) or from the external jugular vein by means of a syringe, and the urine from a catheter kept permanently in the bladder. Probably because of the etherization, the amount of urine obtained was usually small. The experiment was observed for several hours; that is, at least until the glycemia was distinctly on its descending course. The urine was examined at intervals after painting and in some instances the urine excreted during the following 18 or 20 hours was collected and examined. Etheriza-

tion was continued either throughout the entire experiment or until the painting and with it the operative handling of the animal was finished. The quantitative analysis for sugar was made by Myers and Bailey's modification of the Lewis-Benedict method.[3]

After laparotomy the pancreas was exposed to as full a view as possible. At least one-third of the organ was painted. In some experiments the entire quantity of the adrenalin was painted on at once; in others about the same quantity was used but divided for painting several times. The results of the two methods are presented in Tables I and II.

Since the adrenalin will reach the pancreatic cells more thoroughly if applied after the thin membrane and the external covering of connective tissue has been removed, this was done in most of the experiments.

The results will be found in compact but full detail in the tables. Table I presents those obtained in seven experiments in which the pancreas was painted only once with fairly large quantities.

As regards the blood sugar we are concerned essentially with the determinations made during etherization just before exposure of the pancreas and the highest percentage of the sugar found at any time after painting it. The difference between these two determinations indicates the effect of painting the pancreas with adrenalin. Taking the highest increase during an experiment as characteristic of the effect of the painting, the rise in the blood sugar content in the experiments of Table I is as follows: 0.09, 0.07, 0.05, 0.06, 0.00, 0.08, and 0.06 per cent. It varies between 0.09 and 0.00 per cent. The urine never contained reducing substances before painting the pancreas with adrenalin. The highest percentages of glycosuria after painting in the experiments of Table I are as follows: 1.27, 5.00, 0.00, 0.93, 0.68, 0.94, and trace. As can be seen from the data in the table, the percentages of sugar in the urine following the painting were irregular, the highest being 5 per cent and the lowest 0.00 per cent. Evidence of definite relation between glycosuria, hyperglycemia, and the amount of adrenalin used in painting the pancreas is lacking.

Table II summarizes five experiments in which the pancreas was painted several times. The entire quantity of adrenalin used in

[3] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

TABLE I.

Application of Adrenalin to the Pancreas Once.

Experiment No.	Weight. Sex.	Part and proportion of entire pancreas painted.	Amount of adrenalin (1:1,000 solution).	Blood sugar. Before painting. Before ether.	Blood sugar. Before painting. During ether.	Blood sugar. After painting. Time.	Blood sugar. After painting. Percentage.	Urine. Length of period.	Urine. Amount.	Urine. Sugar.	Albuminuria.	Anesthesia.	Temperature of dog.	Remarks.
	kg.		*cc.*	*per cent*	*per cent*	*hrs. min.*	*per cent*	*hrs. min.*	*cc.*	*per cent*			*°C.*	
1	16.4	About one-third (posterior end and body). About one-half of both sides stripped of connective tissue covering.	2.75	0.11	0.12	14	0.15				None before.	Ether (intratracheal insufflation) until 53 min. before last blood sample.	38.2	
						44	0.13							
						→ 1 44	0.21							
	Male.					2 45	0.15		14.5	1.27	+		39.1 38.4	
2	9.25	Entire pancreas. Not stripped of connective tissue covering.	3.0	0.15	0.29	5	0.33				None before.	Ether (intratracheal insufflation) throughout.		Died during night. Duodenum, cecum, and colon intensely hemorrhagic.
						15	0.31							
	Female.					31	0.33						37.5	
						1 1	0.33	1 3	10.5	4.65			36.6	
						2	0.34							
						3	0.36	2 1	15	5.00	+		35.9	
3	17.5	Entire pancreas (posterior end on only one side). Stripped only in one small area.	3.1	0.12	0.16	5	0.21				+ before.	Ether (intratracheal insufflation) throughout.		Died during night. Pneumonia.
						16	0.18							
	Male.					33	0.18							
						1 1	0.18	1	3.2	0.00	++		36.8	
						2 1	0.20						35.9	
						3 2	0.21	2	2.4	0.00			36.6	

4	12.25 Male.	About two-thirds (posterior end and middle). Stripped only in two small areas.	2.4	0.12	0.23	 1 → 2 3 24	6 16 32 1 1	0.27 0.29 0.28 0.28 0.28 0.27 0.13	1 2 21	 About 3 11 >138	 0.0 0.93 0.0	None before. ++ ++ 0	Ether until 2 hrs. after painting (intratracheal insufflation).	37. 35.0 36.7 37.9 39.1	
5	14.5 Male.	About one-third (posterior end and middle). Partly stripped.	2.0	0.13	0.21	 → 2 4 22	9 24 31	0.21 0.14 0.12 0.12	16 2 5 2 20	1.25 15.5 22 >188	0.0 0.68 0.00 0.0	None before. + + + Tr.	Ether by cone for 44 min.	38.5 before. 38.3 38.9 38.5 38.8	
6	7.25 Male.	About two-thirds (anterior end and middle). Not stripped.	2.0	0.12	0.14	 → 2 4	10 19 45	0.22 0.20 0.11	19 2 11 1 50 Post mortem.	A few drops. >10 > 3 21	0.0 0.94 0.0 0.0	+ before. + + +	Ether blown through cone for about 50 min.	38.3	Died during night. Left lung somewhat congested.
7	9.6 Male.	One-third by weight (middle). Not stripped.	1.0	0.13	0.16	 → 2 22	12 54 30	0.22 0.14 0.14	20 2 49 19	4 12 >38	0.0 Tr. 0.0	+ before. + + Tr.	Ether blown through cone for about 39 min.	 39.1 39.3	Vomited while under ether and again afterwards.

In the tables the arrow indicates the time when ether administration was discontinued.

TABLE II.

Application of Adrenalin to the Pancreas Several Times.

Experiment No.	Weight. Sex.	Part and proportion of entire pancreas painted.	Amount of adrenalin (1:1,000 solution).	Blood sugar.				Urine.			Albuminuria.	Anesthesia.	Temperature of dog.	Remarks.
				Before painting.	After painting.									
				Before ether.	During ether.	Time.	Percentage.	Length of period.	Amount.	Sugar.				
	kg.		*cc.*	*per cent*	*per cent*	*hrs. min.*	*per cent*	*hrs. min.*	*cc.*	*per cent*			*°C.*	
8	11.35 Male.	One-half (posterior end and part of middle). Extreme tip stripped.	2.0	0.14	0.28	→ 10	0.30	11	4	4.44	None before. Tr.	Ether by towel for 41 min.	39.0	Vomited while coming out of ether. Pancreas painted twice on one side and three times on the other side.
						2 25	0.21	1 54	13	6.95	"		38.5	
						5 20	0.13	2 54	20	3.55	"			
						23	0.13	17		Tr.(?).				
9	11.05 Female.	One-fourth (posterior free end only). One side stripped.	1.5	0.15	0.24	→ 10	0.29	15	13.5	4.36	Bloody before.	Ether blown through cone for 57 min.	38.8	Each side of pancreas painted twice.
						2 53	0.14	2 35	{ 8	2.70	Bloody.		37.7	
									{ 20.5	3.69	Tr.		38.8	
						4 34	0.13	1 31	26	Tr.	+		39.5	
						22 36	0.12	18		0			40.1	

10	10 Female.	One-third (posterior free end). Stripped to very small extent.	1.5	0.12	0.14	→ 10 2 33 4 32 24 25	0.17 0.12 0.13	15 2 13 3 4 18 50	2 15 25 240	0 Tr. 0 0	Bloody before. Bloody. + + +	Ether blown through cone for 34 min.	39.7 40.3 40.3 39.5	Each side of pancreas painted three times.
11	11.1 Male.	One-third of length (posterior free end). One side stripped.	1.5	0.13	0.24	→ 11 4 20 23	0.33 0.12 0.12	15 3 46 19 30	2.6 23 ?	3.08 1.81 0	Tr. before. ++ +	Ether blown through cone for 49 min.	38.8 38.2 38.0	Each side of pancreas painted three or four times.
12	11.1 Male.	One-sixth of length or one-fourth of weight (part of middle and very little of posterior free end). Both sides stripped.	1.5	0.12	0.18 12 min. later 0.20.	→ 10 2 40 4 35 22 28	0.24 0.13 0.11 0.14	16 2 20 1 55 18	3.6 20 25 ?	3.57 3.07 0.00 0.00	Tr. before. ++ ++ ++ ++++	Ether blown through cone for 50 min.	39.0 38.0 39.0 39.2 38.5	Each side of pancreas painted four times.

the several paintings in each of the five experiments in Table II was generally smaller than those used in the experiments of a single painting in Table I. The rise of the blood sugar in the experiments of Table II is as follows: 0.02, 0.05, 0.03, 0.09, and 0.04 per cent. The highest percentages of sugar in the urine in these experiments were: 6.95, 4.36, trace, 3.08, and 3.57. The increase of the blood sugar is about the same in both tables. The glycosuria generally ran higher in the second series of experiments.

A consideration of both series shows that our experiments bear out in a general manner the statement made by Herter, that in the majority of the cases (nine out of twelve of our animals) painting the pancreas with adrenalin is followed by glycosuria, and further, the amount of the glycosuria varies in different experiments. However, our results do not support unqualifiedly the results presented by Herter and the views expressed by him. As regards glycosuria produced by adrenalin, Herter states that subcutaneous administration produces the least amount, intraperitoneal the next highest amount, and painting the pancreas the highest amount. The impression conveyed is that painting the pancreas will, as a rule, produce high glycosuria, 10 per cent and higher. Thus in one experiment 14 per cent was found, while the slighter glycosuria produced was exceptional and rarely the conditions failed entirely. Now our experiments give rather the opposite impression. Thus in one instance only did the glycosuria approach 7 per cent, while in another it was about 5 per cent. On the other hand, of the twelve experiments, three gave no glycosuria or a mere trace. In seven experiments the glycosuria was small or at least not high. The average of the twelve experiments was 2.23 per cent. In other words, we found the glycosuria following painting of the pancreas with adrenalin a less striking phenomenon and we are not convinced that it differs from the similar condition which follows the subcutaneous or intraperitoneal injection of the drug. Two more points must be considered. First, we employed larger amounts of adrenalin than apparently did Herter and his coworkers. Second, in most experiments we removed the connective tissue and the thin membrane from the part of the pancreas which was to be painted with adrenalin, a device which according to Herter[4] is "apt to be most effective."

[4] Herter, C. A., and Wakeman, A. J., *Tr. Assn. Am. Phys.*, 1902, xvii, 577.

The experiments we have made would seem not to support the chief contention of Herter that the "pronounced nature of the glycosuria following intraperitoneal injections appears to be mainly attributable to the adrenalin which comes into contact with the pancreas."

Although Vosburgh and Richards[2] adopt the view of Herter that the glycosuria produced by injection of adrenalin into the peritoneal cavity is of pancreatic origin, they do not mention the point in the conclusion. As already stated, these authors studied only the effects of adrenalin upon glycemia. They made only three experiments, using large, toxic doses of the adrenalin solutions intraperitoneally; one animal died in 24 hours. We wish now to compare the hyperglycemia obtained in our experiments by painting the pancreas with the results obtained by Vosburgh and Richards. Their Table I contains five such experiments of which the main facts are reproduced in our Table III.

The blood sugar rises in their experiments are: 0.102, 0.118, 0.280, 0.240, and 0.052 per cent. Except in one experiment (No. 7) the rise in the blood sugar is therefore considerably higher than that obtained in our twelve experiments. The average increase in our experiments is 0.054 per cent; the average increase in those of Vosburgh and Richards is 0.158 per cent; thus the average of our experiments is about one-third that of Vosburgh and Richards. The doses of adrenalin employed in four of their experiments were larger than those in ours. Analysis shows, however, that the quantity of adrenalin employed by them did not exert an unmistakable effect upon the blood sugar. Thus in Experiment 4, in which 4 cc. of adrenalin were used, the increase amounted only to 0.118 per cent, while in Experiment 6, in which only 2 cc. were used, the rise was 0.240 per cent. Other points of difference between the methods employed by Vosburgh and Richards and by ourselves exist. The amount of blood withdrawn for each analysis is one of these points. Vosburgh and Richards undoubtedly have withdrawn much larger quantities of blood than we have withdrawn in our experiments. Possibly this is a contributing factor in their finding higher blood sugar than we found.[5] The other possibility of a deeper anesthesia and some de-

[5] For example, see Rinderspacher, K., *Biochem. Z.*, 1910, xxvii, 67–72.

TABLE III.

Blood Sugar Content in Five Experiments on Painting the Pancreas with Adrenalin from Table I of Vosburgh and Richards (Abbreviated).

Experiment No.	Time when blood was withdrawn.	Sugar.	Remarks.
		per cent	
3	3.00 p.m. Normal	0.112	Etherization continued throughout experiment; 3 cc. of adrenalin chloride solution (1: 1,000) applied to pancreas with brush at 3.08 p.m.
	3.15 " 7 min. after painting	0.182	
	3.23 " 15 " " "	0.178	
	3.38 " 30 " " "	0.188	
	4.08 " 1 hr. " "	0.204	
	5.06 " 2 hrs. " "	0.214	
	6.00 " 3 " " "	0.165	
4	7.55 p.m. Normal	0.173	Etherized from 7.45 p.m. till end of experiment; 4 cc. of adrenalin chloride solution (1: 1,000) painted on surface of pancreas at 8.12 p.m.
	8.17 " 5 min. after painting		
	8.27 " 15 " " "	0.277	
	8.42 " 30 " " "	0.237	
	9.12 " 1 hr. " "	0.291	
	10.12 " 2 hrs. " "	0.256	
5	10.45 a.m. Normal	0.239	Ether given throughout experiment; 3 cc. of adrenalin chloride (1: 1,000) applied at 10.55 a.m.
	11.00 " 5 min. after painting	0.291	
	11.10 " 15 " " "	0.354	
	11.25 " 30 " " "	0.388	
	11.54 " 1 hr. " "	0.433	
	12.54 p.m. 2 hrs. " "	0.477	
	1.54 " 3 " " "	0.519	
	2.55 " 4 " " "	0.465	
6	4.52 p.m. Normal	0.131	Ether given throughout experiment; 2 cc. of adrenalin chloride solution (1: 1,000) applied to pancreas at 5 p.m.
	5.05 " 5 min. after painting	0.205	
	5.15 " 15 " " "	0.217	
	5.30 " 30 " " "	0.264	
	6.00 " 1 hr. " "	0.315	
	7.00 " 2 hrs. " "	0.371	
7	3.34 p.m. Normal	0.154	Ether given throughout experiment; 3 cc. of adrenalin solution applied to pancreas at 3.43 p.m.
	3.49 " 6 min. after painting	0.192	
	4.01 " 18 " " "	0.173	
	4.22 " 39 " " "	0.191	
	4.58 " 1 hr., 15 min. after painting	0.206	
	5.43 p.m. 2 hrs. after painting	0.143	
	6.37 " 3 " " "	0.169	

gree of asphyxia (factors which tend to increase the amount of blood sugar) is eliminated by the statement of Vosburgh and Richards that "care was taken to keep the anesthesia as light and as constant as possible." The differences in the method of analysis employed by Vosburgh and Richards (precipitation by phosphotungstic acid and determination of sugar by the Allihn method), and by ourselves (Lewis-Benedict method) cannot be responsible for the variations in our results.

However that may be, we are justified in pointing out the fact that the production of hyperglycemia by painting the pancreas with adrenalin in our experiments was not of unusual degree. There is reason to doubt that the glycosuria and the increase in the hyperglycemia thus produced are greater than would have been produced by the application of adrenalin to any other part of the peritoneal cavity. We have not established by direct experiments the degree of hyperglycemia and glycosuria which is produced by painting some other surface of the peritoneal cavity which would have entitled us to a direct comparison between painting the two surfaces.

On the other hand, we have ascertained definitely that the hyperglycemia and glycosuria produced by intraperitoneal injection of adrenalin is not of pancreatic origin. We have isolated in two series of experiments a part of the pancreas in such a way (1) that the adrenalin applied to the pancreas could not find its way to other parts of the peritoneal cavity at all or to any extent, and (2) that the adrenalin injected into the peritoneal cavity could not reach the isolated part of the pancreas. Table IV records the experiments in which the isolated pancreas was painted with adrenalin.

In the experiments in which the adrenalin was painted on the pancreas and was prevented from coming in contact with any other part of the peritoneum the rise of blood sugar was: 0.02, 0.04, 0.02, 0.06, 0.02, 0.01, 0.04, and 0.07 per cent, giving an average of 0.035 per cent, which is about two-thirds that obtained when the adrenalin was applied to a pancreas remaining in contact with the rest of the peritoneum. The glycosurias were: 0, 1.08, 0, 0, 2.66, 0.57, 0.7, and 0 per cent, with an average of 0.63, which is less than one-third the average of the glycosurias obtained when the painted pancreas remained unisolated from the peritoneum. These figures indicate that the increases

TABLE IV.

Pancreas Isolated; Adrenalin Applied to Pancreas.

Experiment No.	Weight. Sex.	Part and proportion of entire pancreas painted.	Amount of adrenalin (1:1,000 solution).	Blood sugar. Before painting. Before ether.	Blood sugar. Before painting. During ether.	Blood sugar. After painting. Time.	Blood sugar. After painting. Percentage.	Urine. Length of period.	Urine. Amount.	Urine. Sugar.	Albuminuria.	Anesthesia.	Temperature of dog.	Remarks.
	kg.		*cc.*	*per cent*	*per cent*	*hrs. min.*	*per cent*	*hrs. min.*	*cc.*	*per cent*			*°C.*	
											None before.			
13	13.45	One-third (posterior free end).	2.0	0.10	0.11	4	0.12					Ether blown		Free end of pancreas put through a hole in rubber membrane which protected the viscera. Vessels leading to tip ligated. Each side painted six times.
						17	0.13	18	4.4	Tr.	+	through	38.6	
	Male.	Both sides stripped.				→ 4 20	0.11	3 57	75	"	+	cone for 63	39.7	
						22	0.10	17 44	>140	0	+++	min.	38.8	

												None before.			
14	8.75	One-seventh (posterior free end).	1.5	0.13	0.17	→	10	0.21	11	2.4	Tr.	+	Ether blown	38.9	No blood vessels tied. Pancreas brought outside peritoneal cavity as a loop and other viscera protected from adrenalin flowing on them, by several layers of rubber tissue. Each side painted about seven times.
						2	15	0.12	1 53	6.2	1.08	+	through	38.2	
	Male.	Both sides stripped.				4	7	0.12	2	15.4	0	+	cone for 73	39.4	
						22	50	0.11	18 50	>100	0	+	min.	39.5	
												None before.		38.4	
15	10.75	One-fifth (posterior free end).	1.5	0.11	0.13	→	10	0.15	11	1.8	0	+	Ether blown	38.6	The procedure same as in No. 14. Each side painted six or seven times.
						2	32	0.11	2 15	44	0	+	through	38.5	
	Male.	Both sides stripped.				4	18	0.11	1 45	60	0	+	cone for 45 min.	39.2	

TABLE IV—*Continued.*

Experiment No.	Weight. Sex.	Part and proportion of entire pancreas painted.	Amount of adrenalin (1:1,000 solution).	Blood sugar. Before painting. Before ether.	Blood sugar. Before painting. During ether.	Blood sugar. After painting. Time.	Blood sugar. After painting. Percentage.	Urine. Length of period.	Urine. Amount.	Urine. Sugar.	Albuminuria.	Anesthesia.	Temperature of dog.	Remarks.
	kg.		*cc.*	*per cent*	*per cent*	*hrs. min.*	*per cent*	*hrs. min.*	*cc.*	*per cent*			*°C.*	
16	12.0	One-fifth (posterior free end).	1.5	0.12	0.15	→ 11	0.21	13	1.4	0	None before. +	Ether blown through cone for 64 min.	38.7	The procedure same as in No. 14. Each side painted three times.
	Male.	Both sides stripped.						1 52	10	Tr.	+		37.9	
						4 52	0.12	2 36	27	0	+		38.8	
17	11.5	One-third (posterior free end).	1.5	0.09	0.18	→ 10	0.20	14	1.2	About 1	None before. +	Ether blown through cone for 58 min.	39.5 37.1	The procedure same as in No. 14. Each side painted four or five times. Vomited during experiment. Pulmonary edema and pneumonia.
	Male.	Stripped very little.				1 24	0.13	58	9	2.66	+		38.5	
						19 25	0.13	18	>500	0	Tr.		39.0	

18	9.5 Male.	One-half (posterior free end and part of middle). Stripped well.	1.5	0.12	0.25	→	11	0.26		16	3	0.0	None before.	Ether blown through cone for 66 min.	39.2 37.4 38.3 37.0	Procedure similar to that for No. 14, except that the duodenum, wrapped in rubber tissue, was also brought outside. Each side painted three times. Dog dying next morning.
						2	16	0.16	1	55	16.4	0.57	+			
						3	53	0.13	1	35	13	Tr. (?).	+			
						21	40	0.10	18		552	0.0	+			
19	12.5 Male.	One-third (posterior free end and part of middle). Stripped very little.	2.0	0.12	0.21	→	14	0.25		33	4	About 0.7	+ before.	Ether blown through cone for 73 min.	37.0 38.2 38.6 38.9	Procedure similar to that for No. 18, except that other abdominal viscera were still better protected. Each side painted four times.
						3	17	0.12		50	60	Tr.	+			
						5	10	0.12	1	52	55	0.0	Tr.			
						23	30	0.15	18		642	0.0	"			
20	11.35 Male.	One-half (posterior free end and part of middle). Stripped well.	2.0	0.13	0.20	→	10	0.27		21	1.5	0.0	+ before. Bloody.	Ether blown through cone for 55 min.	36.1 38.1 37.3	Procedure similar to that for No. 19. Each side painted four times.
						2	39	0.12	2	20	2.8	0.0	+			
						4	44	0.10	2	5	3.4	0.0	+			
						22	40	0.10	18		>1,000	0.0	+			

TABLE V.

Pancreas Isolated; Adrenalin Administered Intraperitoneally.

Experiment No.	Weight. Sex.	Amount of adrenalin (1:1,000 solution).	Blood sugar. Before injection. Before ether.	Blood sugar. Before injection. During ether.	Blood sugar. After injection. Time.	Blood sugar. After injection. Percentage.	Urine. Amount.	Urine. Sugar.	Anesthesia.	Remarks.
	kg.	*cc.*	*per cent*	*per cent*	*hrs. min.*	*per cent*	*cc.*	*per cent*		
21	11.5	2.0	0.12	0.26	15	0.32	1.4	About 1	Ether blown through cone for 3 hrs., 42 min.	Pancreas and duodenum lifted out of peritoneal cavity by loops of tape and wrapped in cloths soaked in warm saline solution, adrenalin injected below.
					35	0.29	9.8	5.33		
	Female.				1 3	0.23	8.4	5.33		
					2 4	0.22	3.4	6.00		
					→ 3 4	0.16	4.0	2.85		
					3 39	0.13	4.0	1.48		
22	9.3	2.0	0.12	0.23	15	0.27			Ether blown through cone for 3 hrs.	Procedure similar to that for No. 21. Vomited while under ether.
					46	0.27	0.8	0		
	Female.				→ 1 46	0.28				
					2 46	0.18	1.0	0		
23	10.35	2.0	0.11	0.18	15	0.16			Ether blown through cone for 2 hrs., 7 min.	Procedure similar to that for No. 21.
					45	0.13	5	Tr.		
	Male.			30 min. later 0.15	→ 1 47	0.12	A few drops.	0.5 or less.		
					2 47	0.11	1	0.5 " "		

in glycosuria and hyperglycemia observed after intraperitoneal injections are not of pancreatic origin, the effects of the painting being much smaller when the organ is isolated from the peritoneum. This conclusion becomes even more evident when one examines the results of the few experiments given in Table V, in which adrenalin was injected intraperitoneally while the pancreas was isolated.

The rise in blood sugar in this table is: 0.06, 0.05, and 0.01 per cent, the average being 0.04 per cent. The glycosurias are: 6.0, 0.0, and 0.5 per cent, the average being 2.2 per cent. The number of these experiments is too small to permit of a definite decision, yet the conclusion is in harmony with that drawn from the experiments of Table IV; namely, that the hyperglycemia and glycosuria observed after intraperitoneal injections are not of pancreatic origin.

For the sake of clearness the averages of the amounts of urine and of blood sugar noted in Tables I to V are presented in Table VI.

TABLE VI.

Averages of Glycosuria and Rise of Blood Sugar Due to the Application of Adrenalin.

Averages.	Mode of application.	Glycosuria.	Blood sugar
		per cent	*per cent*
Our 12 experiments (Tables I and II).	Painting unisolated pancreas.	2.23	0.054
Our 8 experiments (Table IV).	Painting isolated pancreas.	0.63	0.035
Our 3 experiments (Table V).	Intraperitoneal injections; pancreas isolated.	2.2	0.04
Vosburgh and Richards' 5 experiments (Table III).	Painting unisolated pancreas.		0.158

DISCUSSION AND CONCLUSIONS.

After Blum's discovery of the production of glycosuria by the subcutaneous injection of adrenal extract, Herter has the merit of having found that injection of adrenalin into the peritoneal cavity also produces glycosuria; this is an undeniable fact. Concerning Herter's claim that intraperitoneal injection gives a higher degree of glycosuria

than subcutaneous or intravenous injection, we offer no comment since we have made no observations on the glycosuric effect of subcutaneous injection of adrenalin, while we have made only three experiments by intraperitoneal injection. The most we can predicate on the basis of the present experiments is that intraperitoneal injection of adrenalin produces a somewhat higher degree of glycosuria than could be anticipated. However, in an earlier study carried out several years ago[6] we arrived at the conception that the more slowly adrenalin was absorbed from the tissues into the circulation, the greater was its glycosuric effect; hence an intramuscular injection, which in its effect is nearly equal to that of an intravenous injection, induced a glycosuria definitely smaller than that set up by a similar dose administered subcutaneously. Unless the absorption from the peritoneal cavity is shown to be different from the absorption from subcutaneous injections, there could be no reason to assume that the glycosuric effect of intraperitoneal injection is much greater than that of subcutaneous injection. We might add that our former experiments do not support Herter's view that subcutaneous injection of adrenalin yields only slight degrees of glycosuria, because it is largely oxidized before entering the circulation. A difference exists in the effects upon blood pressure and upon sugar production, depending upon the mode of administration of adrenalin. With regard to the sugar production, a subcutaneous injection has a definitely greater effect than an intravenous injection; with regard to the blood pressure effect, however, the opposite is true. Herter states that an intraperitoneal injection of adrenalin exerts a smaller effect upon blood pressure than an intravenous injection—a fact which Auer and Meltzer can confirm for the rabbit.[7]

Our experiments lead us to conclusions which do not conform to those of Herter. It will be recalled that Herter and his coworkers state first, that painting the pancreas causes a marked glycosuria and hyperglycemia, and, second, that the glycosuria and hyperglycemia produced by intraperitoneal injections are of pancreatic origin; that is, they are produced by the adrenalin's coming in contact with the pancreas. In our experiments tabulated in Table IV, in which the

[6] Kleiner, I. S., and Meltzer, S. J., *J. Exp. Med.*, 1913, xviii, 190.

[7] Auer, J., and Meltzer, S. J., unpublished observations.

pancreas was isolated from the rest of the peritoneal cavity, the glycosuria was about one-third, and the rise in blood sugar about two-thirds that obtained by painting the unisolated pancreas. Hence two facts may be deduced: first, that the painting of the isolated pancreas produces only mild glycosuria and hyperglycemia, and, second, that the greater production of sugar observed after the painting of the unisolated pancreas cannot be of pancreatic origin. Indeed, our experiments point rather to the conclusion that the larger production of sugar after painting the unisolated pancreas is due to the fact that a large part of the adrenalin escapes to the peritoneum. The last mentioned view is supported by the statement of Herter and Wakeman[8] that "applications to the kidney are apt to yield more sugar than similar application to the liver, intestine, spleen, or brain, but the glycosuria is less marked than after the pancreas has been painted." Emerson and one of us had shown that a dissolved substance painted upon a kidney with an intact membrane is incapable of penetrating the membrane and affecting the kidney, or even incapable of entering the circulation, except when the solution escapes to other parts of the peritoneum.[9] It was this observation which led to the suggestion that the effects observed by Herter of painting the pancreas might have been due to the escape of adrenalin to the celiac ganglion. This point has not been directly tested, but several experiments were performed in which the adrenals were painted with the effect on sugar production apparently as intense as that obtained by painting the unisolated pancreas. However this may be, and whether the production of sugar after painting the unisolated pancreas is due to the escape of adrenalin to some definite organ covered by the peritoneum (celiac ganglion or adrenals) or whether the peritoneum as a whole is responsible for the sugar production, it appears that, when sugar production follows the intraperitoneal injection of adrenalin, it is not of pancreatic origin.

[8] Herter and Wakeman, *Tr. Assn. Am. Phys.*, 1902, xvii, 578, foot-note.

[9] Emerson and Meltzer, S. J., cited in *Tr. Assn. Am. Phys.*, 1902, xvii, 595.

THE SPIROCHETAL FLORA OF THE NORMAL MALE GENITALIA.

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PLATES 30 TO 32.

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An accurate knowledge of the varieties of spirochetal organisms which normally inhabit the smegma or the mucous membranes of the urogenital region has become imperative for the establishment of an etiological relationship between a spirochete and a disease in which the organism may be found in the urine.

The classic work of Inada, Ido, Hoki, and others[1] on the presence of *Leptospira icterohæmorrhagiæ* in the urine of convalescents from infectious jaundice has introduced a new procedure by which the disease may be easily diagnosed, and it is natural that a similar procedure should be followed in the search for an etiological agent in other diseases of infectious origin.

Attention has been directed by Martin,[2] Nankivell and Sundell,[3] and Patterson[4] to the urine in cases of trench fevers. In fact, Nankivell and Sundell early demonstrated minute spirochetes in specimens of urine from soldiers suffering from so called trench fever. Of 26 patients, most of them suffering from a "five-day fever," 99 specimens were examined, with 29 positive findings. Spirochetes were found in 12 out of 15 typical cases, while none of the 8 controls showed a spirochete (Fig. 32). The investigators considered the possibility of contamination of the urine from the smegma or from preputial sources, but it seemed to

[1] Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., Etiology, mode of infection, and specific therapy of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiii, 377.

[2] Martin, C. J., quoted by Nankivell and Sundell.[3]

[3] Nankivell, A. T., and Sundell, C. E., On the presence of a spirochæte in the urine in cases of trench fever, *Lancet*, 1917, ii, 672.

[4] Patterson, S. W., Preliminary note on spirochætes occurring in the urine in cases of "P. U. O.," *J. Roy. Army Med. Corps*, 1917, xxix, 503; Spirochætes occurring in the urine in cases of "pyrexia of unknown origin," *Brit. Med. J.*, 1917, ii, 418.

them improbable that the spirochetes came from these sources, since the urine usually contained the minute spirochetes unaccompanied by the coarse *Spironema refringens*, and in case of contamination the presence of the large varieties was to have been expected. Moreover, in some positive instances no spirochetes could be discovered in the smegma. The occurrence of spirochetes in the urine was not constant, that is, not detectable on successive occasions, but was recurrent at irregular intervals. A certain relation seemed to exist between the appearance of spirochetes in the urine and the height of pyrexia, spirocheturia occurring usually 24 hours after the height of fever. In still other cases they appeared on the 14th to the 16th day. They were actively motile, averaged 8.15 μ in length and 0.3 μ in width, with an average of five curves; *i.e.*, varying from two and a half curves in 5 μ to ten curves in 12.5 μ. The spirals varied in depth. The extremities tapered to sharp points with a flagellum at one or both ends. The organism differed from *Treponema pallidum* in its shortness and its fewer spirals.

Patterson, using the Fontana, Wilmaers-Renaux,[5] and India ink methods, examined specimens of urine from various groups of trench affections, 3 cases of trench nephritis, 1 case of pyelonephritis, with abscess of the lungs, 15 cases of relapsing type of pyrexia of unknown origin, 1 case of myalgia following pyrexia of unknown origin, and 5 cases of appendicitis (?) not yet diagnosed, finding spirochetes with the following features: They were about one to one and a half times the diameter of a red blood corpuscle, very thin, with tapering extremities, some having five to eight more or less regular curves, some being straight, some bowed, or lying in a semicircle (Fig. 33). The spirals were not so fine as those of *Treponema pallidum* or so coarse as those of *Spironema recurrentis*. The organisms took Giemsa's, Leishman's, or Romanovsky's stain poorly but were easily demonstrated by the Indian ink method. Patterson depicts the spirochetes found in the abdominal type of cases as rather closely wound, short, thick forms, and those found in the relapsing type as much more tightly coiled. Little attention was given to control cases.

The main objection to the work of the British investigators has been the possibility of an accidental contamination of the urine by unclean surroundings. Many have insisted upon the necessity of collecting specimens by catheterization. The investigation of Stoddard[6] brought out an unsuspected source of spirochetes in the periurethral as well as the intraurethral region of the male genitalia. After examining 50 healthy soldiers and 50 miscellaneous hospital patients without history or symptoms of relapsing fever (trench fever), Stoddard drew the conclusion that (1) spirochetes are not uncommon organisms in the urethra of men without history or symptoms of relapsing fever; (2) many dif-

[5] Wilmaers, L., and Renaux, E., Quarante-sept cas de Spirochétose icterohémorragique, *Arch. méd. Belges*, 1917, lxx, 115, 207.

[6] Stoddard, J. L., Occurrence of spirochetes in the urine, *Brit. Med. J.*, 1917, ii, 416.

ferent varieties are found; (3) some of the varieties seen are morphologically closely similar to pathogenic varieties; (4) the spirochetes occur so definitely within the urethra that they are an obvious source of contamination in uncatheterized specimens of urine; (5) they are a sufficiently dangerous source of error even in catheterized specimens to deserve attention in careful work; and that finally (6) it is possible that a staining reaction or some other morphological character may be discovered to differentiate microscopically the common and harmless from the pathogenic spirochetes. Of 50 hospital cases 56 per cent showed spirochetes, of which 46 per cent were not *Spironema refringens*. Of 50 American soldiers spirochetes in the urethra occurred in 22 per cent, 2 per cent of which showed *Spironema refringens* also. Films from periurethral parts contained more of the coarse *refringens* type.

The spirochetes found by Stoddard measured from 3.75 to 22 μ, more commonly 6.75 to 9 μ, but 11 μ was not uncommon. They were either moderately thick or extremely slender. The ends tapered or were blunt and formed a hook. The spiral length varied from 0.5 to 1 μ. Flattened and longer spirals also occurred, averaging 1.5 to 3.3 μ and as long sometimes as 4 μ. Stoddard states that a type with about eleven curves in 7.5 μ occurs frequently, but I have not been able to verify this finding. In some cases the spirals were exceedingly close and fine and almost impossible to count. They were often irregular, the deep narrow, deep wide, flat narrow, and flat wide types of spirals sometimes occurring simultaneously in the same organism. The middle portion sometimes had looser coils or none at all. In one film many different varieties were often present, including frequently organisms similar to *Leptospira icterohæmorrhagiæ*.[7]

Obviously it is not simple to interpret what one sees in the rich spirochetal material as described by Stoddard, who sees in it many different varieties, including the leptospira type. But, as this article is intended to show, a critical analysis of the spirochetal flora reduces the number of varieties to not more than three, or at most four; namely, *Spironema refringens*, *Treponema calligyrum*, and *Treponema minutum*, *n. sp.*

Since the time of Schaudinn and Hoffmann a coarse spirochete designated by them *Spirochæta refringens* has been known to inhabit the genital region, but no particular attention has been given to the possibility of the existence of other varieties. It was not until the subject was taken up not only from the morphological but also from the cultural standpoint that some interest came to be attached to these

[7] Noguchi, H., *Spirochæta icterohæmorrhagiæ* in American wild rats and its relation to the Japanese and European strains. First paper, *J. Exp. Med.*, 1917, xxv, 755.

spirochetes. In the present work a strain of *Spironema refringens* was isolated and its morphological and cultural features studied, thus establishing its entity as a species. Later a strain of spirochete (*Treponema calligyrum*) was obtained from a condyloma, which resembled *Treponema pallidum* on the one hand and *Spironema refringens* on the other, being an intermediary organism in its morphological and cultural characteristics. Subsequent observations have led me to regard this particular species as one of the most common varieties that are found in the flora of the smegma or of the urethral region. In fact, *Treponema calligyrum* is more frequently met with than the better known coarse *Spironema refringens*. There is, in addition to these two varieties, another, much smaller spirochete in the genital flora, which will be described in a subsequent paragraph. These three, the minute, medium, and coarse types, constitute the spirochetal flora which at first glance present such a complex aspect.

The smegma and urethral films from six soldiers who were admitted to the Hospital of The Rockefeller Institute for treatment for pneumonia have been examined.[8] The specimens were examined in fresh condition under the dark-field microscope and also as stained preparations. For staining methods Giemsa's stain, Fontana's silver impregnation,[9] and occasionally Benians' Congo red negative im-

[8] These specimens were obtained through the courtesy of Captain Henry T. Chickering.

[9] (*a*) Fix the air-dried film in Solution 1, which consists of

	cc.
Glacial acetic acid	8
Formalin	20
Distilled water	100

for 1 minute and wash well with water. (*b*) Mordant with Solution 2, which consists of

Tannin	5 gm.
Phenol	1 cc.
Water	100 "

for 1 minute over a gentle flame to the point of steaming, then wash thoroughly in water. (*c*) Treat in a 0.25 per cent silver nitrate solution to which one drop of ammonia is added to 40 cc. of the solution. The film turns brown in a few minutes. Wash in water and then (*d*) cover with the mordant and warm it over a flame until it begins to steam. Then wash the film in water and dry.

pression method were employed;[10] also a mordant staining recommended by me for various spirochetes, including *Treponema pallidum*. The method is similar to that advanced by Wilmaers and Renaux, but seems to give a better color value on account of the use of gentian violet instead of fuchsin. The film is fixed in methyl alcohol for 15 minutes, then after being washed in water is covered with a solution of mordant (5 per cent tannin plus 1 per cent phenol) and held over a gentle flame for 1 minute, during which time it begins to steam. It is again washed in running water, covered with a strong aqueous solution of gentian violet to which 1 per cent phenol has been added, and steamed briefly over a flame, then washed well in water, air-dried, and examined. This method gives excellent results also with the *pallidum*. Care must be taken not to make too thick a film.

The number of cases examined was small, but the finding was such that it was sufficient to determine the average flora in male genitalia. The varieties of spirochetes encountered in most of the smegma were the same as those found in one. All contained *Treponema calligyrum* and *Treponema minutum, n. sp.*,[11] and most of them *Spironema refringens*, although the latter was absent in some cases.

No spirochetes were found in the films made from the urethral mucosa by means of a platinum loop. Just where the fault in the technique lay I am unable to explain. The finding was uniformly negative also with the specimens of urine from ten soldiers. With the idea that in nephritis cases there might be more possibility of encountering spirochetes in the urine, ten different specimens from acute as well as chronic cases of nephritis were subjected to a careful examination, but with no positive finding as yet.[12] In Table I

[10] A few drops of a 2 per cent Congo red solution (filtered) are mixed with a drop of the material suspected of containing a spirochete and spread over a clean slide to form a film. The slide after being air-dried is immersed in a jar of absolute alcohol containing 1 per cent hydrochloric acid. In a few minutes the red color of the film turns to a bluish tint. The slide is then removed from the acid alcohol and air-dried.

[11] Noguchi, H., Morphological characteristics and nomenclature of *Leptospira* (*Spirochæta*) *icterohæmorrhagiæ* (Inada and Ido), *J. Exp. Med.*, 1918, xxvii, 575.

[12] The specimens used in these tests were obtained through the courtesy of Dr. W. W. Palmer of the Presbyterian Hospital.

are recorded some of the results obtained in the present study. There are at least three different varieties distinguishable in the photomicrographs or under the dark-field microscope, a minute (*minutum*), a medium (*calligyrum*), and a large (*refringens*) type. Their biometric characteristics, as encountered in twenty-five specimens of each type, are given in Table I.

TABLE I.

Type.	Length.		Thickness.	Spiral amplitude and intervals.	Spiral depth.	No. of spirals or waves.
	Average.	Extremes.				
Minute type.	7–10μ	3–14μ	0.25–0.3μ	0.9–1μ. Fairly regular intervals.	0.2–0.5μ. Some may reach 1μ in penultimate spirals.	7–10 spirals; vary according to length.
Medium type.	9–12μ	4–14μ	0.35–0.4μ	1.75μ. Usually fairly regular; that is, a given amplitude is well maintained in a specimen.	0.5–1μ. Often flattened near the middle in stained specimens. Reaches 1.5μ in some.	5–8, varying according to the spiral amplitude; some only 3.
Large type.	12–16μ	7–22μ	0.7μ	2–3μ. Usually more or less regular.	0.5–1.5μ. Almost constant; in live specimens changing the position of the waves. In stained specimens often irregularly flattened out.	3–5; quite variable; exceptionally 8 in a very long specimen.
T. pallidum.	8–14μ	6–18μ	0.25–0.3μ	1μ	0.8–1μ	8–14; some 16.

Some of the dark-field, as well as the ordinary photomicrographs, representing the minute, the medium, and the large types are shown in Figs. 1 to 14. The minute type is decidedly smaller than *Treponema pallidum* and has a larger number of shallower spirals in proportion to its length (Figs. 1 and 5). There are also many short specimens such as are never found among the *pallida*. The medium type has an aspect like that of atypical specimens of the *pallidum*. The spirals are fairly deep but not so deep as those of a typical *pallidum*, while the intervals between them are wider (Figs. 2, 5, 6, 9, and 10). All appear somewhat thicker than the *pallidum* (Fig. 21) when seen under the dark-field microscope. This does not apply to the specimens stained by mordanting techniques (Fontana's and the writer's), in which there occurs often an uncontrollable uneven heavy deposit of the dyes, due to various external factors (Figs. 5 to 14, 17, 20, and 22). Among organisms of the medium type are noticed two forms, one with more closely set spirals and the other with wider ones, but this is due to certain temporary conditions and may be made to disappear or reappear by regulation of cultural conditions. For example, there will be more of the wide, flat spiral forms when the medium is more fluid. The large type is much heavier, comparatively short, with few spirals, and constantly changes its curves (Figs. 4 and 8). The spirals of the minute type become readily obliterated after the death of the organism (upper organism in Fig. 8).

When fresh they all exhibit moderately active movements, rotary, lashing, and forward and backward locomotion. The large type is the most energetic and the minute variety the least so. In many of the large type there is a distinct double contour effect upon examination under the dark-field microscope. All are provided with a terminal filament or flagellum at one or both ends.

As has been noted before, not all smegmata contain a spirochete, and the varieties present may all belong to one or two of the three groups. As a rule, however, all three types are present, the medium type usually predominating.

Cultural Characteristics.

By selecting the smegma specimens which were rich in the type desired, a culture of each of the three types described was obtained. The technique employed was similar to that previously used for the cultivation of *Spironema refringens*[13] and *Treponema calligyrum*.[14] All require strict anaerobiosis (addition of fresh tissue to the media), the presence of suitable body fluid (ascitic fluid), and an optimal temperature (37°C.). The growth in the fluid medium, consisting of ascitic fluid and a piece of fresh rabbit kidney and a layer of paraffin oil, is invisible, while in a solid medium, consisting of 2 parts of the neutral agar and 1 part of ascitic fluid with a piece of the fresh rabbit kidney at the bottom, a faint haze appears to develop near the tissue, gradually extending upward within a fortnight. No discrete, circumscribed, sharp colonies have so far been observed. In this respect all the strains obtained are analogous to the cultures of various anaerobic treponemata and spironemata.[15] None produced a putrefactive or offensive odor, the absence of odor from the culture of the minute type serving to distinguish it from either *Treponema microdentium*[16] or *Treponema mucosum*.[17] Carbohydrates added to the culture media exert neither a favorable nor a retarding influence upon growth, and no visible alterations of the media result from their presence.

In young fluid cultures, whether of the minute, medium, or large type, the organisms are short and active, but as they grow older (2 weeks) the longer forms, some in chains, and some in tangled masses, predominate, their motility meanwhile being considerably reduced. The spirals are quite regular (Figs. 23 to 31). Very short forms do

[13] Noguchi, Pure cultivation of *Spirochæta refringens*, *J. Exp. Med.*, 1912, xv, 466.

[14] Noguchi, Cultivation of *Treponema calligyrum* (new species) from condylomata of man, *J. Exp. Med.*, 1913, xvii, 89.

[15] Noguchi, Experimental research in syphilis with especial reference to *Spirochæta pallida* (*Treponema pallidum*), *J. Am. Med. Assn.*, 1912, lviii, 1163.

[16] Noguchi, Cultural studies on mouth spirochætæ, *Treponema microdentium* and *macrodentium*, *J. Exp. Med.*, 1912, xv, 81.

[17] Noguchi, *Treponema mucosum* (new species), a mucin-producing spirochæta from pyorrhea alveolaris, grown in pure culture, *J. Exp. Med.*, 1912, xvi, 194.

not appear in the solid media, the organisms appearing to attain average length within a short time. The spirals are remarkably regular in solid media and so deep, in the case of the medium type, as to simulate a *pallidum* (Fig. 29). In older cultures two, three, and four individuals in chains have occasionally been encountered (Fig. 27). Division in all three types is brought about by transverse and perhaps also by longitudinal fission.

Identification.

The morphological and cultural characteristics of the large type show it clearly to be a *Spironema refringens*, those of the medium type identify it with *Treponema calligyrum*. The latter type may be the same organism as that described by Levaditi and Stanesco[18] in 1909 as *Spirochæta gracilis*, found in a case of balanitis, but, as pointed out previously, these authors used a name already designating another spironema from an ulcerating jaw, which is very different from the present medium type. The name *Treponema calligyrum* was given to a non-pathogenic spirochete cultivated from the surface of a condyloma, but subsequent studies on the spirochetal flora of the genitalia have convinced me that this type is one of the most commonly met inhabitants of the genital region.

The minute type is not unlike the minute spirochete of the mouth, *Treponema microdentium*, but its cultural characteristics differentiate it from the latter. *Treponema microdentium* produces a peculiar odor, especially when freshly isolated, and in a fluid medium the color of the fresh tissue is made grayish within about 10 days and the fluid somewhat faintly opalescent. The *minutum* produces no odor and remains without any perceptible action upon the culture medium, though in dimension there is a general resemblance.

In order to determine whether these two closely similar organisms are immunologically related to each other, agglutination tests were undertaken in which the action of a *microdentium* antiserum (rabbit) was tested on both types. It was found that the serum caused a marked agglutination of *Treponema microdentium* in 1:500 dilution but only a slight one with two different strains of the *minutum*, even

[18] Levaditi, C., and Stanesco, V., Culture de deux spirochètes de l'homme (*Sp. gracilis* et *Sp. balanitidis*), *Compt. rend. Soc. biol.*, 1909, lxvii, 188.

in a dilution of 1:20. In this connection it may be mentioned that a *calligyrum* serum (rabbit) gave a copious agglutination with the cultures of the medium type in a 1:200 dilution, but only a slight one with those of the minute type in 1:20. There was a partial reaction, but not marked enough to render the differentiation of the two types difficult.

In all probability the minute smegma spirochete has been repeatedly observed by investigators, but no special attention seems to have been given to its identity. I have been accustomed to pass it over as probably identical with *Treponema microdentium*. Now that this type has been found to constitute an independent group, differentiated by several well defined features, it may well be known under a separate name, *Treponema minutum*.

In the spirochetal flora of male smegma, therefore, only the three forms, *Spironema refringens*, *Treponema calligyrum*, and *Treponema minutum*, were recognized.

DISCUSSION AND SUMMARY.

The varieties of spirochetes enumerated and photomicrographed from the male smegma flora represent practically every form hitherto described by Nankivell and Sundell and by Patterson in the specimens of urine from trench fever cases (Figs. 32 and 33). The urethral flora, as studied by Stoddard, seem to contain more varieties, but, except those of his more detailed morphological descriptions, every form observed by him is among those found in the smegma. Stoddard saw certain forms with hooked ends suggestive of the *Leptospira icterohæmorrhagiæ* of infectious jaundice, but the resemblance ends with this one feature, and differentiation should always be possible under the dark-field microscope, by means of which the leptospira reveals its highly characteristic minute elementary spirals, presenting the appearance of a chain of dots (Fig. 18). Fig. 19 shows that a very favorable fixation with the osmic acid vapor followed by Giemsa's staining may also bring out the elementary spirals. Of all the spirochetes, none has so closely set spirals as the jaundice leptospira, the distance between two spirals being only 0.5 μ. Various methods, including Fontana's, Benians', the mordant gentian violet stain, or Burri's India ink method, are inadequate

to differentiate the leptospira from other spirochetes (Figs. 12, 14, 15, 16, 17).

Why a positive spirochete finding with the films from the urethra and in the specimens of urine was not obtained, is difficult to explain, except on the grounds of the paucity of specimens examined. At all events, the recent negative results reported by Fiessinger[19] with French soldiers and invalids after cleansing of the urethra and glans seem to be in harmony with my results.

In conclusion it may be stated that *Spironema refringens*, *Treponema calligyrum*, and *Treponema minutum* represent practically all the spirochetal forms observed in the male smegma flora. A leptospira has never been conclusively shown to be present in the specimens of normal urine or smegma. For the satisfactory microscopic demonstration of a leptospira a dark-field illuminator is indispensable.

EXPLANATION OF PLATES.

PLATE 30.

Magnification, × 1,000.

FIGS. 1 to 4. Dark-field views of the spirochetes in a male smegma. Fig. 1 represents *Treponema minutum*, Fig. 2 *Treponema calligyrum*, Fig. 3 *Spironema refringens*, and Fig. 4 a *Spironema refringens* (below) and a *Treponema minutum*.

FIGS. 5 to 11. Various types of spirochetes in smegma, stained by Fontana's method.

FIG. 5. Two specimens of *Treponema minutum*.

FIG. 6. A specimen of *Treponema minutum* and two of *Treponema calligyrum*, of varying lengths.

FIG. 7. A group of *Treponema calligyrum*, with two specimens of *Treponema minutum*.

FIG. 8. A group of *Spironema refringens* from a sample of male smegma.

FIGS. 9 to 11. *Treponema calligyrum* from two different specimens of male smegma.

FIGS. 12 and 13. *Treponema calligyrum* in preparation stained by the mordant gentian violet method. In Fig. 12 there are two specimens without distinct spirals which closely resemble *Leptospira icterohæmorrhagiæ* in similar stained preparations. Further study by means of a dark-field microscope is necessary to determine whether they are leptospira or *calligyrum*.

FIG. 14. A group of *Treponema calligyrum* type from a specimen of male smegma, stained by Giemsa's method. The organisms appear much thinner here than in specimens stained by other methods. A hooked spirochete resembling strongly the leptospira is seen near the left upper corner.

[19] Fiessinger, N., À propos des Spirochètes du méat et de l'urine de l'homme normal, *Compt. rend. Soc. biol.*, 1918, lxxxi, 38.

FIGS. 15 to 19. *Leptospira icterohæmorrhagiæ* under various conditions (for comparison).

FIG. 15. Four specimens of *Leptospira icterohæmorrhagiæ* stained by the mordant gentian violet method. They appear blunt and curved and without any indication of the minute elementary spirals which are the characteristic feature of this genus. As they appear here they are indistinguishable from the stretched forms of the *calligyrum* type.

FIG. 16. A few leptospiræ as demonstrated by Benians' Congo red method. Here, too, they do not show their elementary spirals.

FIG. 17. A group of *Leptospira icterohæmorrhagiæ* from a culture, stained by Fontana's method. They fail to show their elementary spirals by this staining.

FIG. 18. A leptospira viewed under the dark-field microscope, showing its minute elementary spirals.

FIG. 19. A number of *Leptospira icterohæmorrhagiæ*, fixed with osmic acid vapor and stained by Giemsa's stain, showing the elementary spirals.

FIGS. 20 to 22. *Treponema pallidum* under different conditions (for comparison).

FIG. 20. *Treponema pallidum* when stained by the mordant gentian violet method.

FIG. 21. *Treponema pallidum* under the dark-field microscope.

FIG. 22. *Treponema pallidum* as stained by Fontana's silver impregnation method.

PLATE 31.

Magnification, × 1,000.

FIGS. 23 and 24. Dark-field view of a culture of *Treponema minutum* from a male smegma.

FIG. 25. *Treponema minutum* from a culture. Stained by the mordant gentian violet method.

FIG. 26. Similar specimens stained by Fontana's method.

FIG. 27. Dark-field view of a culture of *Treponema calligyrum* from a male smegma.

FIG. 28. A culture of *Treponema calligyrum* stained by the mordant gentian violet method.

FIG. 29. Similar specimens stained by Fontana's method.

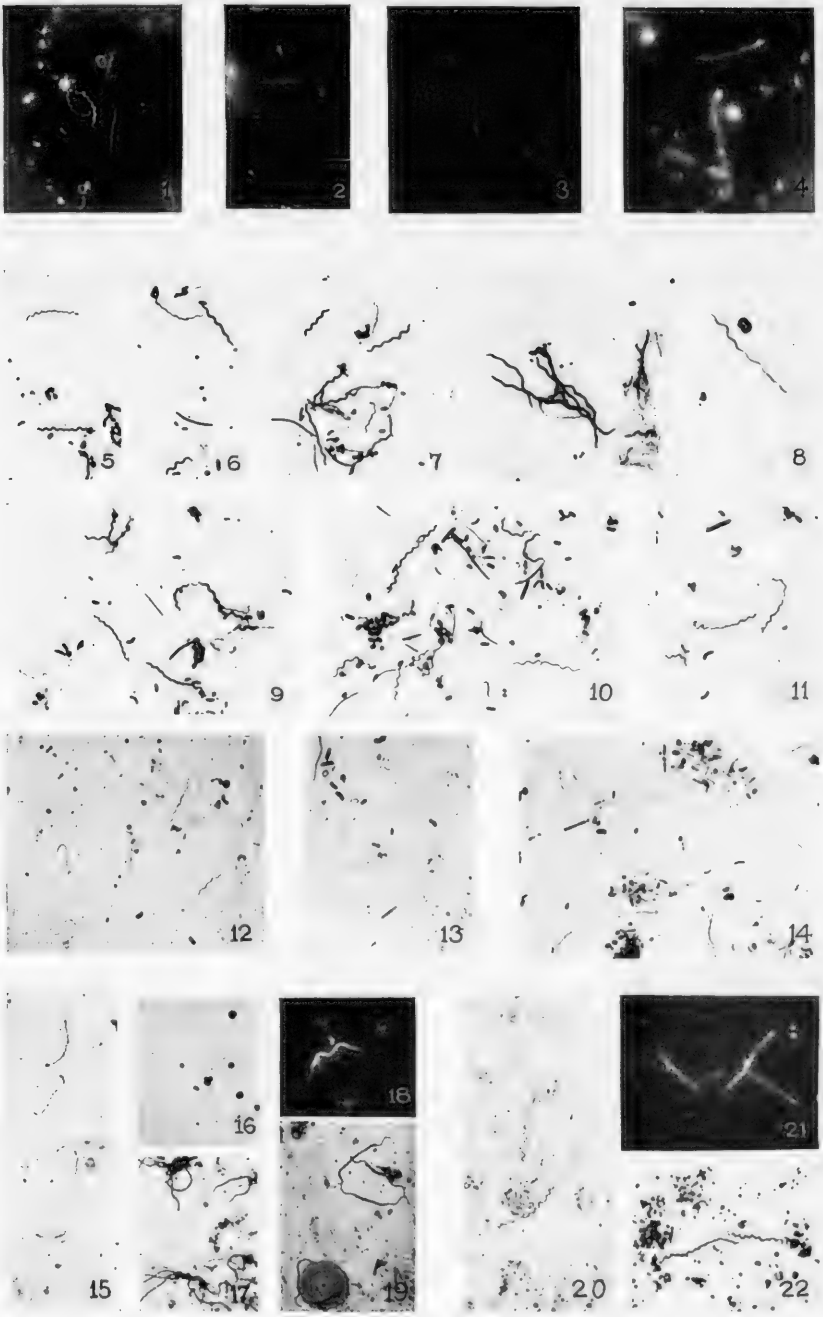
FIG. 30. Dark-field view of a culture of *Spironema refringens* from a male smegma.

FIG. 31. Similar specimens stained by Fontana's method.

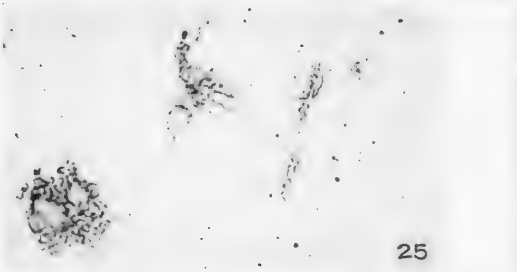
PLATE 32.

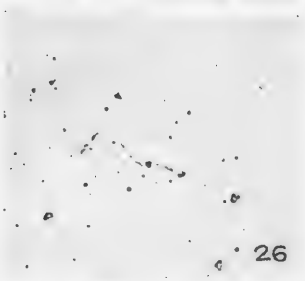
FIG. 32. Photographic reproduction of the photomicrographs of spirochetes in Nankivell and Sundell's article on the spirochetes in the urine in trench fever cases.[3]

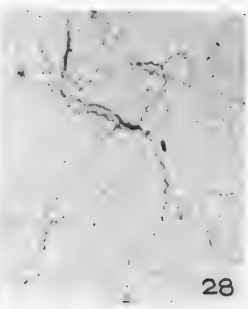
FIG. 33. Photographic reproduction of the schematic drawings by Patterson in his article.[4]

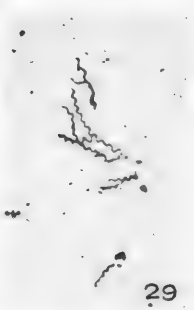


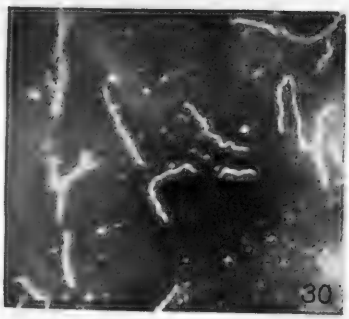
(Noguchi: Spirochetal flora of normal male genitalia.)

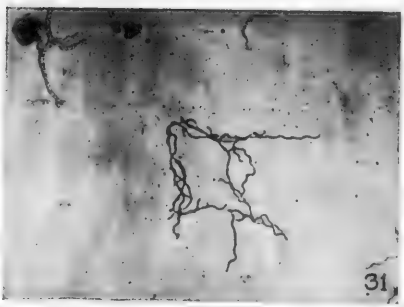












(Noguchi: Spirochetal flora of normal male genitalia.)

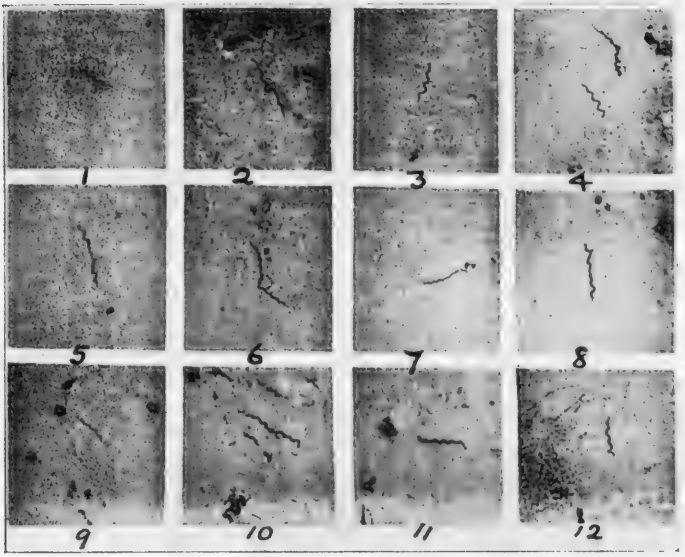
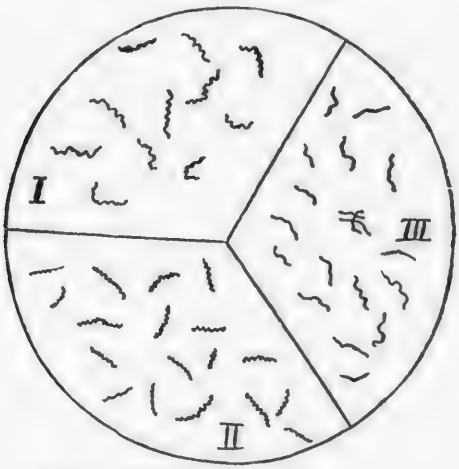


FIG. 32.



I. Spirochetes of Type 1, abdominal P. U. O.
II. Type 2, relapsing P. U. O.
III. Spirillar form from urethra.

FIG. 33.

(Noguchi: Spirochetal flora of normal male genitalia.)

PHYSIOLOGICAL STIMULATION OF THE CHOROID PLEXUS AND EXPERIMENTAL POLIOMYELITIS.

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(Received for publication, March 15, 1918.)

When the active filterable virus of poliomyelitis is injected into the blood of monkeys, infection and paralysis almost never follow even when the quantity of virus introduced is very large. The reason assigned for the non-infectiousness of the virus under these conditions, compared with the remarkable activity displayed by it when brought into immediate proximity with the central or even the peripheral nervous organs, is the inability of the virus to pass the barrier of the choroid plexus and the blood vessels of the central nervous system. Flexner and Amoss[1] have shown in several series of experiments that when sterile irritating chemical substances are introduced from without and by lumbar puncture into the subarachnoid space, the injury inflicted upon the choroid plexus and blood vessels of the meninges and possibly those of the central nervous organs also, facilitates the passage of the virus from the blood into the nervous tissues under conditions leading to infection, paralysis, and death from poliomyelitic disease. Their experiments have led them to view the meningeal-choroidal complex as constituting in man a defensive mechanism against infection with the virus of poliomyelitis.

According to this view, disturbance of the integrity of the defensive complex arising from any cause would predispose to infection with the virus, provided the disturbance synchronized with the wide distribution of the virus, such as is believed to be the case during epidemics of poliomyelitis. The experiments of Flexner and Amoss have indicated that the qualitative changes in the meningeal-choroidal com-

[1] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249; 1917, xxv, 525. Amoss, H. L., and Eberson, F., *ibid.*, 1918, xxvii, 309.

plex, permitting the escape of the virus from the blood into the nervous tissues, may be almost infinitesimally small. Thus the mere substitution of the cerebrospinal fluid of one monkey for that of another sometimes suffices to open this way. The structural alterations induced by this procedure must be so slight as to be regarded merely as molecular; and yet they have proved adequate to overcome the defensive mechanism.

All the means employed up to the present to disturb the mechanism may be regarded as organic in their effect. Moreover, they have always acted from without, in the sense that they have been brought into relation with the meningeal-choroidal complex through the medium of the cerebrospinal fluid already present in the subarachnoid space. The question presented itself whether a functional effect merely and acting, as it were, from within, might likewise open the way for the passage of the virus from the blood into the nervous tissues. A method was at hand to test experimentally this possibility.

The cerebrospinal fluid is a secretion derived from the blood through the mediation of the choroid plexus. The secreting cells of the plexus exercise a highly precise discrimination in respect to the quality and quantity of the constituents taken from the blood and passed on to the subarachnoid space. The composition of the cerebrospinal fluid not only differs markedly from that of the blood, but dissolved drugs and bacteria and their toxic and other metabolic products present in the blood are capable of being excluded perfectly from the cerebrospinal fluid by the choroidal mechanism.

The stimulus on which the secretory activity of the choroid plexus depends has been shown by Dixon and Halliburton[2] to be a hormone contained within the choroid plexus and to a less extent in the brain substance. The liberation of this hormone into the blood is the precursor to and regulating medium of the choroidal secretory activity, through which the cerebrospinal fluid is elaborated. By increasing experimentally the quantity of the hormone within the blood the amount of cerebrospinal fluid secreted within a unit of time may be increased.

[2] Dixon, W. E., and Halliburton, W. D., *J. Physiol.*, 1910, xl, p. xxx; 1913–14, xlvii, 215; 1914, xlviii, 128.

This phenomenon provides, therefore, a means by which the choroid plexus may be stimulated from within and made to perform its secretory function in an intensified manner. It furnishes a simple method for determining whether merely increased functional activity, independent of structural or organic alterations, suffices to open the way for the passage of the poliomyelitic virus from the blood into the central nervous organs under circumstances leading to infection.

The experiments to be described were carried out to determine this point. The procedure followed for preparing and injecting the extract of the choroid plexus was closely modelled on that of Dixon and Halliburton. These investigators found that an intravenous injection of a saline extract of the choroid plexus, after a delay of a few seconds, causes the cerebrospinal fluid to flow actively for a variable time, after which the flow ceases gradually. The second injection produces little or no effect unless the intervening interval of time is about 10 or 15 minutes.

EXPERIMENTAL.

The starting-point of our experiments was a repetition of the decisive experiments of Dixon and Halliburton. The first step was the preparation of an extract of the choroid plexus. 1 gm. of the dried plexus was ground up with clean sand in a mortar in 100 cc. of isotonic saline solution. The suspension was filtered and the filtrate employed for injection. 5 cc. of the filtrate caused a marked increase in the flow of cerebrospinal fluid in a dog weighing 12 kilos. Extracts of the fresh plexus give an equivalent result, and boiling does not destroy the activity. The active substance is, moreover, soluble in dilute and absolute alcohol. An extract of the brain is less active than one of the plexus. Material from the dog, sheep, or ox may be employed. Dixon and Halliburton, who established these points, express the view that some product of the brain's metabolism passes to the choroid plexus and this hormone stimulates to activity the secreting epithelium covering the plexus. They also discuss the possibility of the hormone's originating in the choroidal epithelium and passing secondarily to the brain tissue. They incline to the first alternative.

Preparation of the Choroid Extract. Isotonic.—Plexuses removed from the fresh brains of sheep under sterile conditions are washed free from blood in sterile saline solution, dried between filter paper, weighed, made up to 1 per cent suspension in isotonic saline solution, ground with sand, and filtered.

Hypertonic.—The same steps are followed except that 10 per cent of the dried plexuses are suspended in 8.5 per cent saline solution. The stock solution is then diluted 1 part to 9 of sterile distilled water before injecting. The filtering of the viscous mixture, which is a slow process, may be substituted by rapid centrifugalization. The clear supernatant fluid is removed and diluted as indicated.

Preliminary Tests.

The technique of the experiments was perfected on dogs in accordance with the method devised by Dixon and Halliburton. A single protocol is appended to illustrate a successful experiment.

Oct. 22, 1917. Dog; weight 12.5 kilos. Anesthesia: chloroform, morphine, and urethane (subcutaneous). Subcerebellar cistern punctured. After the first rapid rush of cerebrospinal fluid was over, the flow was measured in drops per minute and total volume for 10 minutes. In this animal the first rapid escape was 8.2 cc. The slower flow is divided into three 10 minute periods: (*a*) before injecting extract and (*b*) (*c*) after injecting two separate quantities of the extract into the left femoral vein.

1st Period. Before Injection of Extract.

Drops per min.	Total in 10 min. cc.
3, 0, 0, 0, 1, 0, 3, 1, 1, 2	0.7
0, 1, 0, 0, 1, 0, 0, 0, 0, 1	0.2

2nd Period. 5 Cc. of Extract Injected into Left Femoral Vein.

Drops per min.	Total in 10 min. cc.
0, 5, 3, 2, 1, 1, 1, 1, 1, 1	1.2
1, 0, 0, 3, 3, 0, 0, 0, 1, 1	0.7
1, 0, 0, 1, 0, 1, 2, 1, 1, 1	0.5

3rd Period. 3.8 Cc. of Extract Injected into Left Femoral Vein.

Drops per min.	Total in 10 min. cc.
2, 1, 0, 1, 1, 0, 1, 1, 1, 1	0.4
1, 1, 0, 0, 1, 0, 0, 0, 1, 0	0.2
0, 0, 0, 0, 0, 0, 0, 0, 0, 1	0.05

The effect of the choroidal extract is observed after each injection, but not so markedly after the second injection.

The preliminary test on monkeys was even more satisfactory. The extract was injected into the basilic vein of a *Macacus rhesus* in an amount of 10 cc. in an animal weighing 3.5 kilos, without producing an observed ill effect. Two protocols of preliminary experiments on monkeys are given.

Oct. 22, 1917. Monkey A, *Macacus rhesus*; weight 5 kilos. Anesthesia: ether, morphine, and urethane. Puncture of subcerebellar cistern. The periods and readings are the same as in the previous protocol. The first flow of fluid following the puncture was 4.4 cc.

1st Period. Before Injection of Extract.

Drops per min.	Total in 10 min. cc.
2, 1, 1, 0, 1, 1, 1, 2, 5, 0	0.8
3, 2, 1, 1, 1, 1, 1, 0, 0, 0	0.5

2nd Period. 3.5 Cc. of Extract Injected into Basilic Vein.

Drops per min.	Total in 10 min. cc.
0, 0, 2, 2, 1, 1, 1, 1, 2, 2	0.9

Oct. 25, 1917. Monkey B, *Macacus rhesus*; weight 3.5 kilos. Anesthesia: ether, morphine, and urethane. The first flow of fluid following the puncture was 4.1 cc.

1st Period. Before Injection of Extract.

Drops per min.	Total in 10 min. cc.
1, 4,* 2, 1, 1, 2, 4,* 3, 1, 1	1.6
2, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.1

2nd Period. 6 Cc. of Extract Injected into Basilic Vein.

Drops per min.	Total in 10 min. cc.
1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0.7
1, 0, 0, 1, 1, 0, 1, 1, 1, 1	0.3

* In the interval represented by the two asterisks ether was administered.

The deduction from the three preliminary experiments is to the effect that the choroidal extract which we prepared and employed was an active one. There is another point which may be mentioned here. In order to obtain the stimulating effect of the extract, it

would appear that the animals need to be in good condition. Two monkeys in an advanced stage of tuberculosis showed no effect from the injections.

Experiments with Choroid Extract.

In carrying out experiments with the extract, the quality of the virus is of prime importance. It must be of such a degree of activity that it will not itself induce infection by simple intravenous injection, and yet it must be active enough to cause infection under conditions in which it is enabled to pass the choroidal-meningeal barrier. The importance of this consideration is illustrated by the first protocols.

Experiment 1.—Control A, *Macacus rhesus*. Dec. 5, 1917. Intravenous injection of 40 cc. of centrifugate of 5 per cent emulsion of fresh mixed virus. Dec. 11. Legs weak or paralyzed; right deltoid weak. Dec. 13. Both legs paralyzed; tremor of head. Dec. 14. Prostrate. Dec. 19. Died.

Autopsy.—Lesions of poliomyelitis.

Monkey C, *Macacus rhesus*. Dec. 5, 1917, 12.50 p.m. Intravenous injection of 40 cc. of centrifugate as in control. 12.55, 1.25, and 4.55 p.m. Intravenous injection of choroid extract. Dec. 6, 11 a.m. and 5 p.m. Injection of choroid extract. Dec. 12. Left facial paralysis and slight ataxia. Dec. 14. Legs paralyzed; deltoids weak. Dec. 17. Arms and back weak; lies down. Dec. 19. Improving. Jan. 2, 1918. Recovering use of limbs. Jan. 8. Recovered except for residual paralysis of legs.

Monkey D, *Macacus rhesus*. Dec. 5, 1917, 1.05 p.m. Intravenous injection of 40 cc. of centrifugate as in control. 1.10, 1.40, and 5.10 p.m. Injection of choroid extract. Dec. 6, 11 a.m. and 5.10 p.m. Injection of choroid extract. Dec. 7 and 8. Injection of choroid extract. Dec. 10. Right facial and double deltoid paralysis. Dec. 11. Died.

Autopsy.—Lesions of poliomyelitis.

This experiment is wholly inconclusive as to any promoting effects of the choroidal extract after an intravenous inoculation of the virus. Since the virus was of so high a degree of activity as to induce a fatal infection in the control monkey, the occurrence of paralysis in the other two animals was to be expected. Moreover, a comparison of Monkeys C and D suffices to dissipate any notion that the choroidal extract might have the effect of minimizing the action of the virus since Monkey C partially recovered from the paralysis. Indeed, this experiment is a pertinent illustration of the factor of individuality

in affecting the outcome of an attack of poliomyelitis in the monkey, as well as in man. This factor of individuality appears even more emphatically in the next experiment.

Experiment 2.—Control B, *Macacus rhesus*. Oct. 29, 1917. Intracerebral inoculation of 1 cc. of 5 per cent emulsion of fresh spinal cord and medulla from paralyzed monkey. Nov. 2. Excited; tremor of head. Nov. 3. Tremor increased; ataxic. Nov. 6. Right facial paralysis; left arm weak. Nov. 8. All limbs paralyzed; moribund; etherized.

Autopsy.—Lesions of poliomyelitis.

Control C, *Macacus rhesus*. Oct. 29, 1917. 50 cc. of centrifugate of fresh emulsion of brain and cord, same as Control B, injected intravenously. Nov. 5. Double facial paralysis; ataxia; paralysis of right deltoid. The paralysis extended rapidly so that by evening the animal was prostrate and death occurred during the night.

Autopsy.—Lesions of poliomyelitis.

Monkey E, *Macacus rhesus*. Oct. 29, 1917, 12.50 p.m. Intravenous injection of 50 cc. of centrifugate, same as Control C. 1, 1.30, and 5 p.m. 5 cc. of choroidal extract injected. Oct. 30, 11 a.m. and 5 p.m. 5 cc. of choroidal extract injected. Oct. 31 and Nov. 1. 5 cc. of choroidal extract injected. Nov. 5 and 6. No extract injected. Nov. 7. Ataxia; limbs weak. Nov. 8. Right facial paralysis; deltoids paralyzed; legs weak. Nov. 10. Prostrate. Nov. 12. Moribund; etherized.

Autopsy.—Lesions of poliomyelitis.

Monkey F, *Macacus rhesus*. Treatment identical with that of Monkey E, except that no intravenous injections of choroidal extract were given after Oct. 30. This animal never showed any symptoms and was dismissed from observation on Nov. 19, at which time it was perfectly well.

The only deduction from this experiment is to the effect that the virus was sufficiently active to cause infection and paralysis in two of three monkeys into which it was injected intravenously while a third monkey was sufficiently insusceptible to resist its power of inducing infection. The choroidal extract probably played no essential part in the results.

In order to determine directly whether the choroidal extract exercised a restraining influence on the development of the infection the next experiment was performed.

Experiment 3.—Control D, *Macacus rhesus*. Dec. 4, 1917. Intraspinal injection of 2 cc. of sterile isotonic saline solution. Dec. 5. Intravenous injection of 40 cc. of centrifugate of fresh mixed virus. Dec. 10. Tremor; ptosis. Dec.

11. Ataxia; left deltoid and right leg weak. Dec. 12. All extremities paralyzed; etherized.

Autopsy.—Lesions of poliomyelitis.

Test: Monkey G, *Macacus rhesus.* Dec. 4, 1917, intraspinal injection of saline solution and Dec. 5, 12.05 p.m., intravenous injection of virus as in the control. 12.10, 12.40, and 4.10 p.m. 5 cc. of choroidal extract injected. Dec. 6. Repeated injection of extract at 10.10 a.m. and 4.10 p.m. Dec. 11. Left facial and right leg paralysis. Dec. 12. Both legs paralyzed; arms weak. Dec. 14. All extremities paralyzed. Progressive recovery followed. Animal regained use of arms. Jan. 18, 1918. Died of intercurrent infection.

Autopsy.—Healed lesions of poliomyelitis.

It is obvious that the course of the infection was practically identical in these two animals, and no inhibitory effect of the choroidal extract can be discerned.

Passing now to a virus which is incapable in the quantity employed of inciting infection from simple intravenous injection, we find that the injection of the choroidal extract does not change the results.

Experiment 4.—Control E, *Macacus rhesus.* Nov. 13, 1917. Intravenous injection of 25 cc. of centrifugate of active fresh virus (emulsion of medulla and spinal cord of paralyzed monkey). No symptoms developed and the animal was dismissed from observation on Dec. 17, at which time it was perfectly well.

Monkey H, *Macacus rhesus.* Nov. 13, 1917, 12.55 p.m. Intravenous injection of 25 cc. of virus as in the control. 1, 1.30, and 5 p.m. Injection of 5 cc. of choroidal extract. Nov. 14, 11 a.m. and 5 p.m. Injection of 5 cc. of choroidal extract. Nov. 15 and 16. Injection of 5 cc. of choroidal extract. No symptoms developed and the animal, in perfect health, was dismissed from attention on Nov. 30.

Experiment 5.—Control F, *Macacus rhesus.* Dec. 18, 1917. Intravenous injection of 32.5 cc. of centrifugate of active fresh virus (emulsion of medulla and spinal cord of paralyzed monkey). No symptoms developed.

Monkey I, *Macacus rhesus.* 12 m. Intravenous injection of 32.5 cc. of centrifugate as in the control. 12.05, 12.35, and 4.05 p.m. 5 cc. of choroidal extract injected. Dec. 19, 10.15 a.m. and 4.05 p.m. 5 cc. of choroidal extract injected. Dec. 20 and 21. 5 cc. of choroidal extract injected. No symptoms developed. The animal was dismissed from observation on Jan. 8, 1918, at which time it appeared perfectly well.

DISCUSSION AND SUMMARY.

The experiments recorded in this paper serve, in the first place, to confirm the experiments of Dixon and Halliburton on the stimulating effect of intravenous injections of extracts of choroid plexus in

the secretion of the cerebrospinal fluid, and extend their observations to monkeys.

They bring out also the variable effects of the virus of poliomyelitis, variations affected by the quality of the virus and also by the individual powers of resistance to infection possessed by individual monkeys. These factors of variation must be taken into account in performing and interpreting experiments on infection and particularly those on immunity and specific therapy in relation to poliomyelitis.

In general it may be said that experimental infection by way of the blood is not easy to produce in monkeys unless some contributing factor, such as the existence of a coincident aseptic meningitis, operates at the same time. And yet Experiments 1 and 2 show that when the strength of the virus is great the injection of relatively considerable quantities suffices to induce infection and paralysis, but not in all instances.

The chief outcome of the experiments has been to determine the fact that when the intravenous inoculation of the virus does not in itself suffice to induce infection and paralysis, the intravenous injection of extracts of the choroid plexus, which in themselves excite the secretory functions which preside over the formation of the cerebrospinal fluid, is powerless to modify this result. This fact would seem to be of interest and importance, since it has already been shown that very slight structural changes in the meningeal-choroidal complex suffice to make possible or certain infection under these circumstances. Apparently mere augmentation, from time to time, of the secretory functions of the choroid plexus, through intravenous injection of an extract of the choroid plexus and while the virus is still circulating, is insufficient to insure passage of the virus from the blood into the nervous tissues, upon which infection depends. Neither does the augmentation exercise a restraining influence on the development of infection otherwise capable of taking place.

THE AUTODIGESTION OF NORMAL SERUM THROUGH THE ACTION OF CERTAIN CHEMICAL AGENTS. I.

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INTRODUCTION.

In contrast with the so called defensive ferment, or "*Abwehrferment*" of Abderhalden, which has been recently much studied and discussed, comparatively little attention has been paid to the proteolytic ferment in normal serum, to which only occasional brief references can be found.

Abderhalden[1] has stated that he sometimes found a proteolytic ferment in the sera of guinea pigs and rabbits, which he held to have arisen through the introduction of foreign proteins, such as those due to the ingestion of plants, or to infectious diseases, especially coccidiosis. Stephan[2] reported that guinea pig serum shows an apparently polyvalent proteolytic power. Fuchs[3] found that rabbits inoculated with serum gave a positive ninhydrin reaction with other kinds of substrates, and he explained this result by assuming that the sera of herbivorous animals contain a comparatively large amount of dialyzable substance. Michaelis and von Lagermarck[4] obtained a positive Abderhalden reaction not only with pregnant serum but also with non-pregnant and even male serum, and they came to the conclusion that they could not confirm the existence of the specific ferment in Abderhalden's sense. Van Slyke, Vinograd-Villchur, and Losee[5] also

[1] Abderhalden, E., Abwehrferment. Das Auftreten blutfremder Substrate und Fermente im tierischen Organismus unter experimentellen, physiologischen und pathologischen Bedingungen, Berlin, 4th edition, 1914, 53–54.

[2] Stephan, R., Die Natur der sogenannten Abwehrfermente, *Münch. med. Woch.*, 1914, lxi, 801.

[3] Fuchs, A., Tierexperimentelle Untersuchungen über die Organspezifität der proteolytischen Abwehrfermente (Abderhalden), *Münch. med. Woch.*, 1913, lx, 2230.

[4] Michaelis, L., and von Lagermarck, L., Die Abderhaldensche Schwangerschaftsdiagnose, *Deutsch. med. Woch.*, 1914, xl, 316.

[5] Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., The Abderhalden reaction, *J. Biol. Chem.*, 1915, xxiii, 377.

found proteolytic ferment in non-pregnant human serum by means of Van Slyke's method of amino nitrogen determination.

The existence, then, in normal human and animal serum, of a non-specific proteolytic ferment which digests certain proteins other than the serum has often been proved, but little investigation into the nature of this ferment has hitherto been made. The question of autodigestion of normal serum has received some attention from a few investigators, Delezenne and Pozerski[6] having observed the autolysis of the serum under the influence of chloroform.

The present paper deals with a phenomenon of the autodigestion of normal serum brought about with certain chemical agents under various conditions.

Materials and Methods of Study.

Guinea pig serum was used chiefly in the present investigation, because it possesses advantage over other sera in its constancy and its richness in the ferment in question. Since, to secure uniformity of results, it was necessary to provide a sufficiently large quantity of serum for each series of experiments, with small animals a pool had to be made of many specimens from animals killed at the same time. When guinea pigs were used, the blood was withdrawn from the heart under general anesthesia by means of a sterile test-tube provided with a sharp cannula. The blood was collected in a sterile paraffined centrifuge tube, and upon coagulation it was centrifuged to separate the serum from the clot. By this method a clear serum, absolutely free from any trace of hemolysis, may be obtained. It is important to note that for the demonstration of autodigestion of normal serum through the intervention of certain chemical substances no specimen which contains hemoglobin should be employed, since, as will be shown later, the presence of hemoglobin and stroma, whether homologous or alien, leads to the appearance of digestive products and renders the issue of the self-digestion of the serum indecisive. The experiments were carried out with fresh active serum, although it was found that the activity of the serum is not perceptibly impaired by standing at a temperature of 6°C. for many days.

The amino substances normally contained in serum were previously removed by dialysis. The serum was placed in sterile celloidin sacs and was allowed to dialyze for 5 hours at room temperature in a sterile salt solution which renewed itself from a flow from another bottle placed above the level of the dialysis vessel. The celloidin sacs were preserved in sterile distilled water with a layer of toluene

[6] Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, lv, 327, cited by Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xix, 460.

and before use were washed repeatedly with sterile salt solution. A layer of toluene protected the serum from bacterial interference during dialysis. The volume of the serum at the completion of dialysis was increased from one and a half times to twice its original volume. To secure a constant concentration the dialyzed serum was diluted with sterile salt solution until the volume became twice that of the original serum; that is, the dialyzed serum was made one-half of the original concentration. The dialyzed serum thus obtained, when kept in the refrigerator at 6°C., does not lose its proteolytic power for a long time, at least not for 3 or 4 weeks. We therefore kept in this way a sufficient supply of serum to complete many successive experiments with the same material.

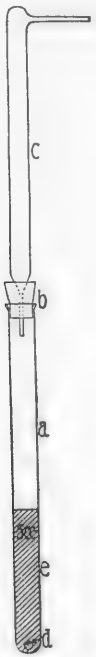
The technique for dialysis was somewhat similar to that recommended by Abderhalden. The dialyzing thimbles used were those made by Schleicher and Schüll bearing the mark of 579A. In order to select perfect thimbles, it was necessary to test beforehand their permeability and intactness by means of solutions of silk peptone (Höchst) and egg white. Those which leaked or showed unusual porosity or retardation of dialysis were discarded as unsuitable. A serum to be tested for digestion was measured into a thimble which stood inside a sterile Jena glass wide mouthed flask. The height of the thimble and that of the flask were about the same, and the former was held upright by the edge of the latter. 15 cc. of sterile distilled water were poured into the flask outside the thimble. At the termination of dialysis, the fluid outside the thimble, representing the dialysate, was removed for determination of the amount of dialyzable proteins diffused out of the serum contained within the thimble. For this purpose the ninhydrin reaction was resorted to.[7] Since this reaction requires a temperature above 100°C. maintained for at least 1 minute, it was not easy to obtain a uniform and constant result, owing to rapid evaporation and frequent loss of the fluid incidental to the violent bubbling caused by the application of a direct flame to the test-tube containing the dialysate and ninhydrin solution. A few previous workers have attempted to eliminate errors arising from this source by using a liquid paraffin bath instead of a direct flame. The overboiling of the fluid from the test-tube placed in the paraffin oil bath at a temperature above 100°C. was greatly reduced by a specially devised stopper,[8] but we have found this device of little value, since it fails to prevent the loss of fluid by explosive escape of vapor, which forces out the fluid gathering about the narrow exit for steam.

After experiments with various devices a satisfactory result was obtained with the use of one suggested by Dr. Noguchi and illustrated in Text-fig. 1. With this

[7] The Van Slyke apparatus for the determination of amino nitrogen was also used in certain series of experiments where the amounts of the split products were sufficiently large to use this apparatus, but in ordinary experiments the amounts were too minute to permit its use.

[8] Oeller, H., and Stephan, R., Technische Neuerungen zur Dialysiermethode, *Deutsch. med. Woch.*, 1913, xxxix, 2505.

apparatus only occasionally does a small amount of the fluid escape. It consists of a hard glass test-tube (Pyrex), 1 cm. in diameter and 20 cm. in height (*a*), connected through a perforated rubber stopper (*b*) with another, somewhat narrower test-tube (*c*), the mouth of which is drawn into a long narrow neck to fit the stopper, and which has a narrow side arm near the bottom. When connected, the smaller test-tube, with narrow openings at both ends, stands in-



TEXT-FIG. 1. Apparatus used for the ninhydrin test. *a*, test-tube connected through a perforated rubber stopper (*b*) with another, narrower test-tube (*c*). In the lower test tube is a glass bead (*d*) which facilitates uniform diffusion of heat during the boiling of the mixture of dialysate and ninhydrin solution (*e*).

verted. The stopper may be lifted out with the upper tube attached and the mixture (*e*) of the dialysate (5 cc. as a rule) and ninhydrin solution (1 cc. of a 1:1,000 solution for 5 cc. of dialysate) placed in the lower test-tube, with a glass bead (*d*), which facilitates uniform diffusion of heat during the boiling. The upper portion is then tightly refitted, and the fluid is ready for boiling. The paraffin oil bath is made by filling an enameled pan with a sufficient amount of

the oil to give a depth of about 12 cm., which will cover nearly two-thirds of the height of the lower test-tube containing the fluid for heating. The bath should have width enough to hold a metal rack containing several tubes, as it is a great advantage to heat the entire series of tubes used in the experiment at the same time. It may be mentioned that the heating period is an important factor in relation to the intensity of the ninhydrin reaction. The color which manifests itself on cooling is gradually increased as the heating period is prolonged, although it was impossible to ascertain definitely, on account of the rapid evaporation of the fluid, at what rate and how long the increase proceeded. It was found, however, that the reaction at the end of 1 minute was much weaker than that of 5 minutes, and that after 10 minutes much stronger than that of 5 minutes' duration. A comparison of the intensity of the reaction was, of course, made after the volume of the fluid had been restored to the original standard by adding distilled water to the 5 cc. mark in the tube. It is easily seen, therefore, that a reaction which increases in intensity through minute errors due to inaccurate time limits would be greater during the preliminary few minutes than at the end of 5 minutes or longer. For this reason, throughout the entire experiment, instead of the 1 minute period of other investigators, we heated the fluid for exactly 5 minutes at a temperature of 150°C., or as near 150°C. as possible, the temperature being maintained by means of an oil bath in a wind-proof hood. At the end of 5 minutes the tubes were taken out of the bath and left at room temperature for 30 minutes before the reaction was read. In order to obtain a uniform and comparable result the content of each tube, which was reduced almost one-half through evaporation, was filled with distilled water up to the original volume of the dialysate; namely, 5 cc. in our experiment. The intensity of the reaction varied from a mere nuance to a distinct violet, with many intermediate grades. It was therefore necessary to prepare a standard by which different degrees of the reaction could be determined. Alanine was selected for producing the required color reaction by ninhydrin. 0.01 cc. of this substance, in 0.1 N solution, gives a distinct violet color, while 0.0025 cc. gives only a faint violet, when present in 5 cc. of distilled water. It was therefore possible to prepare a series of tubes in which color scales, based upon the gradually increasing amounts of 0.1 N alanine solution, were obtainable.

In the present study the reaction produced by 0.01 cc. of a 0.1 N solution of alanine in 5 cc. of distilled water was chosen as the standard. In an estimation of color intensity there may be two procedures. One is to have many grades of the color for comparison with a given reaction. The other, which is the one adopted in the present work, is to have one standard and to estimate the intensity of a given reaction by noting the amount of distilled water necessary to reduce the color exactly to correspond with the standard. If a given reaction requires a quadruple dilution to reach the standard, its intensity must be considered quadruple the standard; a reaction requiring triple or double dilution would be triple or double in strength.

For practical purposes, we have arbitrarily designated the reaction ++++ when the standard was attained by diluting with 3 to 3.9 cc. of water, +++ with 2 to 2.9 cc., ++ with 1 to 1.9 cc., and + with 0.9 cc. or less. Reactions weaker than this were recorded as <+ and ±, which corresponded with a mixture of 1 cc. of the standard and water up to 1 cc. and that containing more than 1 cc. of water, respectively. The reactions may be briefly summarized as follows:

++++ for a reaction requiring 3 to 3.9 cc. of water to make it correspond with the standard.

+++ for a reaction requiring 2 to 2.9 cc. of water.

++ for a reaction requiring 1 to 1.9 cc. of water.

+ for a reaction requiring 0 to 0.9 cc. of water.

<+ for a reaction corresponding with standard 1 cc. + water up to 1 cc.

± for a reaction corresponding with standard 1 cc. + water more than 1 cc.

The ninhydrin reaction with amino-acid undergoes, within a day or so, a rapid discoloration, which cannot be prevented even by preserving the tubes in a refrigerator at 6°C. A suitable substitute was sought, therefore, among various violaceous aniline dyes, and it was found that a certain high dilution of crystal violet resembles very much the ninhydrin reaction, when carefully adjusted to the standard color of the latter, and remains unaltered for a long time, provided it is kept in a dark refrigerator. The standard color solution of crystal violet was utilized for the reading of the reaction because of its stability. It should be added, however, that when subjected to further dilution, the relative color values and effect no longer run parallel.

The proteolytic activity of the serum was tested not only for the autodigestion caused by chemical reagents, but also by using as substrates some pure preparations of plant or animal proteins and various animal tissues or blood corpuscles. When animal tissues were used, they were freed from blood, boiled, and emulsified exactly as in the procedure recommended by Abderhalden. All these substrates were dialyzed in a celloidin sac before use in order to remove any dialyzable protein substances which might be contained in some of the preparations.

Occurrence of the Proteolytic Ferment in Normal Guinea Pig Serum.

To 2 cc. of the dialyzed guinea pig serum various substrates, as shown in Table I, were added and digested in thimbles for 16 hours at 37°C. The control tests done with each substrate alone gave no color reaction, whereas those done with 2 cc. of dialyzed guinea pig serum gave a reaction of only ±.

As will be seen from the table, the guinea pig serum, when incubated with some animal and plant proteins, produces dialyzable substances which show a positive ninhydrin test. Whether the serum in this case really digested the substrates, or whether the former was

TABLE I.

Effect of the Proteolytic Ferment of Normal Guinea Pig Serum on Different Substrates.

Substrate boiled.	Ninhydrin test.	Substrate boiled.	Ninhydrin test.	Substrate boiled.	Ninhydrin test.
Guinea pig liver.	+++	Rabbit placenta.	+++	Cat serum.	±
" " corpuscles.	+++	" serum.	±	" fibrin.	±
" " placenta.	+++	" fibrin.	±	Sheep corpuscles.	+++
Chicken liver.	+++	Dog corpuscles.	+++	" fibrin.	±
" corpuscles.	+++	" serum.	±	Egg white.	±
" serum.	±	" fibrin.	±	Casein (Hammersten).	+++
Rabbit liver.	+++	Cat liver.	+++	Edestin (Merck).	+++
" corpuscles.	+++	" corpuscles.	+++	Ricin (Merck).	++
Guinea pig serum.	±	Dog liver.	+++	Sheep serum.	±

brought to autodigestion only by the influence of the substrates, is not shown by this experiment. The question will be discussed in more detail below. Among the substrates tested, the serum and fibrin of various animals and egg white remained indifferent to the proteolytic ferment of serum. The presence of such a polyvalent proteolytic ferment in normal serum is already known.

Autodigestion of Normal Serum through the Action of Certain Chemicals.

Quite distinct from the proteolytic phenomenon already described is the autodigestion of normal serum brought about through the intervention of non-nitrogenous chemicals such as acetone, alcohols, and chloroform. Table II gives the results obtained when these

TABLE II.

Autodigestion of Normal Serum as a Result of Treatment with Certain Chemical Reagents.

Test No.	Dialyzed guinea pig serum.	Chemical reagents.	Digested.	Ninhydrin test.
	cc.			
1	2.0	Acetone (Kahlbaum) 0.8 cc.	In thimble at	+++
2	2.0	Methyl alcohol (Kahlbaum) 1.0 cc.	37°C. for 16	+++
3	2.0	Chloroform (Kahlbaum), shaken, 2.0 cc.	hrs.	+++
4	2.0	Salt solution 1.0 cc.		±

chemicals were added to the dialyzed guinea pig serum, and the mixture was incubated at 37°C. for 16 hours. This phenomenon seems to suggest a sort of activation of the serum ferment by these chemicals.

The object of the following experiment was to determine the optimal concentration of acetone for a given volume of serum in order to cause autodigestion. The dialyzed guinea pig serum (2 cc.) was mixed in test-tubes with 1 cc. of acetone of various concentrations. After standing for 30 minutes at room temperature, the contents of each test-tube were transferred into a thimble and digested at 37°C. for 16 hours. The dialysates outside the thimbles were tested with ninhydrin (Table III).

TABLE III.

Optimal Concentration of Acetone to Activate Serum.

Test No.	Dialyzed guinea pig serum.	Salt solution.	Acetone.	Concentration of acetone mixture.	Ninhydrin test.
	cc.	*cc.*	*cc.*	*per cent*	
1	2.0	0	1.0	$33\frac{1}{3}$	−
2	2.0	0.3	0.7	$23\frac{1}{3}$	+++
3	2.0	0.5	0.5	$16\frac{2}{3}$	+
4	2.0	0.7	0.3	10	±
5	2.0	0.8	0.2	$6\frac{2}{3}$	±
6	2.0	0.9	0.1	$3\frac{1}{3}$	±
7	2.0	1.0	0	0	±

As will be seen from the table the optimal concentration of acetone is very limited, and, according to repeated tests, it lies between 23 and 28.5 per cent. Either a lower or a higher concentration than this causes less effect, and no digestion takes place beyond a certain point. An amount of acetone which is sufficient to produce a strong turbidity or precipitation in the serum destroys the serum ferment at the same time, and there is no means of securing an active ferment by precipitating it from the serum with acetone. The same is true when the serum is precipitated with alcohol. The extreme lability of the serum ferment against acetone and alcohol presents a striking contrast to pepsin, trypsin, and other ferments, which, as is well known, withstand treatment with these reagents.

In autodigestion the serum no doubt plays the part of the ferment solution as well as of the substrate; hence, the more serum is used,

ceteris paribus, for digestion, the more split products are to be found in the dialysate. The relation of varying amounts of serum to digestion, under constant acetone concentration, was considered in the next experiment (Table IV). The result shows that under the same conditions of total liquid volume and acetone concentration the concentrated serum solution produces more dialyzable substances than the diluted one.

TABLE IV.

Relation between Various Amounts of Serum and the Degree of Autodigestion under Constant Acetone Concentration.

Test No.	Dialyzed guinea pig serum.	Salt solution.	Acetone.	Ninhydrin test.
	cc.	*cc.*	*cc.*	
1	2.0	0.2	0.8	+++
2	1.0	1.2	0.8	+
3	0.5	1.7	0.8	<+
4	0.25	1.95	0.8	±
5	0.1	2.1	0.8	−
6	0.05	2.15	0.8	−
7	2.0	1.0	0	±

Influence of Higher Temperature on the Serum Protease.

The test-tubes, each containing 2 cc. of the dialyzed guinea pig serum, were placed in the water bath regulated at 37°, 55°, and 60°C., respectively. After 30 minutes the tubes were taken out of the baths and allowed to cool at room temperature. An adequate amount of acetone or substrate (boiled chicken liver) was then added to the serum to permit detection of the presence of the ferment (Table V). The proteolytic ferment of serum, as the result shows, survives exposure to 55°C. for 30 minutes, but it is completely destroyed by heating at 60°C. for 30 minutes. The serum to which a suitable amount of acetone or a substrate had been added did not undergo autodigestion when placed in the incubator at 55°C. The optimal temperature for the action of this ferment seems to be 37°C.

TABLE V.

Resistance of the Serum Protease to Temperature.

Test No.	Dialyzed guinea pig serum.	Temperature applied.	Further treatment.	Ninhydrin test.
	cc.	°C.		
1	2.0	37	Acetone 0.8 cc.	+++
2	2.0		Substrate.	+++
3	2.0		Salt solution 0.8 cc.	±
4	2.0	55	Acetone 0.8 cc.	+++
5	2.0		Substrate.	+++
6	2.0		Salt solution 0.8 cc.	±
7	2.0	60	Acetone 0.8 cc.	±
8	2.0		Substrate.	±
9	2.0		Salt solution 0.8 cc.	±

Digestion Experiment in Test-Tubes without Simultaneous Dialysis.

In the preceding experiment autodigestion proceeded simultaneously with dialysis, since the serum and substrates or chemical activators were placed in a dialyzing thimble from the beginning. The question naturally arose whether or not the rate of digestion would be equally great if the mixture were put in a test-tube instead of a dialyzing thimble. There was reason to think that certain chemical activators such as acetone or alcohols would exert in test-tubes an injurious effect upon the serum ferment when added in proportions optimal for a dialyzing thimble, because in the latter a continuous reduction of the chemicals through osmosis must constitute a factor for yielding a maximum hydrolysis. In other words, the amounts of the reagents for digestion in thimbles would be too large for an optimal action of the ferment in test-tubes. This proved to be the case, as may be seen from the experiment recorded in Table VI.

Test 1 is a control test, showing the digesting power of the serum alone, without any treatment. Test 2 is another control, which demonstrates positive autodigestion caused by acetone. Test 3 shows that the serum loses its proteolytic power when mixed with acetone and kept at a temperature of 37°C. for 30 minutes. Test 4 shows that the acetonized serum which stands at room temperature for 30

minutes and then at 37°C. for 30 minutes is also inactivated. Test 5 shows that inactivation also takes place when the serum is acetonized immediately after being taken out of the water bath. Test 6 shows that a previous incubation of the serum for 30 minutes at 37°C. has no injurious effect upon the ferment action if acetone is introduced after the serum has been sufficiently cooled by standing 30 minutes after the bath.

The foregoing experiments indicate that the quantity of acetone inducing an optimal digestion of the serum in a dialyzing thimble destroys the ferment in 30 minutes when the mixture is kept at 37°C.,

TABLE VI.

Effect of Acetone upon the Serum Protease in the Test-Tube at Different Temperatures.

Test No.	Dialyzed guinea pig serum 2 cc. in test-tube.					Transferred into thimble; digested at 37°C. for 16 hrs.
	At 37°C. in water bath.	At room temperature.	Acetone.	At room temperature.	At 37°C. in water bath.	Ninhydrin test.
	min.	*min.*	*cc.*	*min.*	*min.*	
1						±
2		30	0.8	30		+++
3			0.8		30	±
4			0.8	30	30	±
5	30		0.8	30		±
6	30	30	0.8	30		+++

while no injurious effect can be detected when it is kept at room temperature for half an hour.

That the use of the dialyzing thimble is an important factor in attenuating the destructive action of acetone upon the ferment through rapid exosmosis of the reagent is shown by the presence in the dialysate of some acetone soon after dialysis began. Prevention of exosmosis of acetone from the dialyzing thimble by the addition to the outside water of acetone in exactly the same proportion as that contained in the serum within the thimble results in total inactivation of the serum protease, as will be seen from the following experiment.

In Test 7, 2 cc. of dialyzed guinea pig serum were placed in a thimble with 0.8 cc. of acetone. Instead of the usual distilled water 15 cc., a mixture of dis-

tilled water 10.7 cc., and acetone 4.3 cc., was placed outside the thimble. The concentration of acetone was then equal on both sides of the thimble. As was expected, at the end of the usual incubation period, no digestion was found to have taken place.

Unlike acetone, chloroform and tissue substrates exert no injurious action upon the ferment, even when employed in excessive quantities; hence autodigestion by means of these substances can be carried out in test-tubes.

Removal of the Activating Reagents from the Mixture with Serum.

The phenomenon of autodigestion of serum through the intervention of certain reagents, belonging chiefly to the group of so called fat solvents, arouses interest as to the causes underlying this interaction. With acetone and the simpler alcohols it was noticed that a faint opalescence appears when the reagents are mixed with serum in the optimal proportion. Whether or not this slight physical change has any relation to autodigestion is not apparent. Moreover, in the case of chloroform, which is an excellent activator, no perceptible change, except the emulsification of the serum, takes place. One might assume that autodigestion is brought about by the extraction of fatty and lipoidal substances from the serum proteins, thus enabling the serum protease to act upon the delipolyzed proteins. But ether, benzene, toluene, or petroleum ether, in spite of their delipolyzating powers, have no activating property. At all events, it seemed important to ascertain what would happen if the chemicals once mixed with the serum were extracted from the mixture. As will be shown in the following experiments, it was found that serum once acetonized or treated with other suitable chemical activators in proper proportions remains autodigestive even after the activators are completely removed. The continued presence of the activating reagents in the serum is not necessary in order to induce autodigestion.

The chemical activators can be eliminated from the mixture with serum either by (1) evaporation, (2) dialysis, or (3) treatment with other indifferent substances which free the serum from the activating chemicals.

Evaporation Method.—Evaporation by means of vacuum is preferable, because it can be done at a lower temperature and with the least risk of bacterial contamination. A temperature above 15°C. should be avoided, since, in the mixture with activators, the activity of the ferment is highly sensitive to higher temperatures.

A mixture of serum with an adequate amount of a chemical activator is put into a large sterile Petri dish, the cover replaced, and the whole placed in a desiccator, which is then exhausted by means of vacuum. As soon as the pressure drops below a certain point, the

TABLE VII.

Effect of the Removal of Acetone from the Serum Mixture by Evaporation in Vacuo.

Test No.	Dialyzed guinea pig serum.	Acetone.	Further treatment.		Digested in thimble. Dialysate.	
					Test for acetone.	Ninhydrin test.
	cc.	*cc.*				
1	2.0	0	Controls. No further treatment.		−	±
2	2.0	0.8			++	+++
3	2.0	0.8	Acetone evaporated to the point when bubbling ceased. Volume restored to 2 cc.		+	+++
4	2.0	0.8		Acetone 0.8 cc.	++	+++
5	2.0	0.8	Acetone completely removed by desiccation *in vacuo*. Volume restored to 2 cc.		−	+++
6	2.0	0.8		Acetone 0.8 cc.	++	+++
7	2.0	0.8		Boiled.	−	−

contents of the dish begin to bubble. During the bubbling care must be taken to avoid loss of the liquid by overflow by regulating the speed of evaporation. The liquid ceases to bubble in a few minutes, as much of the activating reagents is already driven out of the mixture, but the odor reveals the presence of the small amount remaining. For complete removal of the reagents, evaporation must be continued until the contents of the dish are quite or nearly dried up. The residue obtained is then redissolved in sterile distilled water of a volume equal to that of the original dialyzed serum.

Acetone, chloroform, and methyl alcohol can be easily removed by this method, on account of their having a lower boiling point than the higher alcohols. But the higher series of alcohols, having a higher boiling point than that of distilled water, cannot be satisfactorily eliminated by this method.

TABLE VIII.

Effect of the Removal of Chloroform from the Serum Mixture by Evaporation in Vacuo.

Test No.	Dialyzed guinea pig serum.	Further treatment.			Digested in thimble. Ninhydrin test.
	cc.				
1	2.0	Control. No further treatment.			±
2	2.0	Shaken with chloroform repeatedly; stood at room temperature for 30 min.			+++
3	2.0		Chloroform completely evaporated. Volume restored to 2 cc.		±
4	2.0			Shaken again with chloroform.	+++
5	2.0	Shaken with chloroform repeatedly; stood at 6°C. for 30 min.			+++
6	2.0		Chloroform completely evaporated. Volume restored to 2 cc.		+++
7	2.0			Shaken again with chloroform.	+++
8	2.0			Boiled.	–

The behavior of the serum ferment after it has been freed from its activators by evaporation is shown in Tables VII and VIII. These tables show that acetone and chloroform can be completely removed from the mixture without any loss in the autodigestive activity of the serum, since it had already been activated by the reagents (Table VII, Test 5; Table VIII, Test 6). There is, however, a slight difference between the two reagents in their mode of action. The activating action of acetone is rather rapid, while that of chloro-

form is much slower (Table VIII, Test 3), requiring nearly 2 hours to insure an activation which will endure after the evaporation of the chemical.

Dialysis Method.—This method can be used only for the elimination of water-soluble substances, such as acetone and the lower alcohols. It is unavailable for chloroform and certain higher alcohols which are insoluble or less soluble in water. In order to utilize

TABLE IX.

Effect of the Removal of Acetone from the Serum Mixture by Evaporation and Dialysis.

Test No.	Dialyzed guinea pig serum.	Acetone.	Further treatment.			Digested in thimble. Dialysate.	
						Ninhydrin test.	Test for acetone.
	cc.	*cc.*					
1	2.0	0	Controls. No further treatment.			±	–
2	2.0	0.8				+++	++
3	2.0	0.8	Acetone partly evaporated immediately after having been mixed with serum.			+++	+
4	2.0	0.8		Residual acetone removed by dialysis for 2 hrs.		±	–
5	2.0	0.8			Acetone 0.8 cc.	+++	++
6	2.0	0.8	Acetone partly evaporated after standing with serum for 30 min. at room temperature.			+++	+
7	2.0	0.8		Residual acetone removed by dialysis for 2 hrs.		+++	–
8	2.0	0.8			Acetone 0.8 cc.	+++	++

celloidin membrane for dialysis it was necessary to remove, by a brief preliminary evaporation *in vacuo*, much of the reagent from the mixture, as the presence of acetone in such a concentration may affect the membrane. Complete removal of the reagent is then effected by dialysis in celloidin sacs for 2 hours in running salt solution. This combined method was used for the serum employed in Table IX.

As Test 4 shows, digestion cannot take place when the acetone is removed immediately after being mixed with the serum. If removal is begun after the mixture has already been allowed to stand at room temperature for 30 minutes, however, there is no difference in the ultimate outcome (Test 7). It is therefore advisable, in order to insure a thorough activation, to keep the mixture of serum and acetone at room temperature for at least 30 minutes before further treatment is started.

Extraction with Indifferent Fat Solvents.—As indifferent substances for the removal of chloroform or acetone from the serum by extraction, ether and petroleum ether were used, since they were found to possess neither an activating nor an injurious effect upon the serum

TABLE X.

Effect of Extraction by Means of Petroleum Ether of the Acetonized Serum.

Test No.	Kind of serum.	Further treatment.	Digested in thimble. Ninhydria test.
1	Extracted serum 2 cc.	Alone.	+++
2		Acetone 0.8 cc.	+++
3		Emulsion of residuum 0.5 cc.	+++
4	Unextracted dialyzed guinea pig serum 2 cc. (controls).	Alone.	±
5		Acetone 0.8 cc.	+++
6		Emulsion of residuum 0.5 cc.	±

ferment. It was understood from the beginning that even by repeated and renewed extractions the acetone or alcohols cannot be completely exhausted from the serum admixtures. However, a point of interest in this mode of extraction lies in the fact that by it not only the added chemicals, but also the native fats and lipoids are removed, as is not the case in the evaporation or dialysis methods. Methyl alcohol is far less amenable to extraction from its mixture with serum, either by ether or by petroleum ether. An experiment in which this method was used follows.

4 cc. of acetone were mixed with 10 cc. of dialyzed guinea pig serum in a large centrifuge tube. After the mixture had been standing for 30 minutes at room temperature 10 cc. of petroleum ether were added to the liquid, which was then shaken energetically. The

emulsified liquid was centrifuged and the clear upper layer, consisting of petroleum ether and acetone, separated with a pipette. The extraction procedure was repeated five times and the extracted serum subsequently placed in a vacuum apparatus in order to remove the petroleum ether. The portions of petroleum ether containing fractions of acetone and representing several renewed extractions were reunited and evaporated *in vacuo*. The residue was emulsified in 1 cc. of salt solution (Table X).

As far as the experiment is concerned, the extraction of the acetone from the acetonized serum with petroleum ether makes no difference in the digesting process (Test 1). In other words, the absence of the substances of serum soluble in petroleum ether and acetone has no influence on the autodigestion of serum. It is interesting to note further that the addition of the lipoidal emulsion had neither an inhibitory action nor an accelerating influence upon the ferment activity of either the extracted (Test 3) or the unextracted (Test 6) serum. There was no antiferment in this fraction against the serum protease in question.[9]

Influence of Reactions upon the Serum Protease.

It is well known that the activity of a ferment is greatly influenced by the reaction of the medium in which it is found. In order to ascertain the optimal reaction for the serum protease, experiments were performed in which the digestion of the serum was carried out in various reactions. For this purpose amounts ranging from 0.01 to 1 cc. of a 0.1 N solution of hydrochloric acid or sodium hydroxide were added to a number of test-tubes, each containing a mixture of dialyzed guinea pig serum 2 cc., and acetone 1 cc. The total volumes of the mixtures were made uniformly 4 cc. by adding salt solution in the necessary amounts. The mixtures were allowed to stand at room temperature for 30 minutes and then were transferred

[9] The inactivity of the lipoid and fatty constituents of serum as an antiferment is attributed by Jobling and Petersen (*J. Exp. Med.*, 1914, xix, 549) to an imperfect dispersion after they are once extracted. By saponification they found them to be highly antienzymic. It seems open to discussion whether the antienzymic property of an unsaturated soap can explain the original antiferment of the serum.

to a corresponding number of dialyzing thimbles for incubation. The thimbles were placed in dialyzing flasks containing distilled water to which such quantities of acid or alkali were added as would make the reaction correspond exactly with the acidity or alkalinity of the contents of each thimble. The digestion was continued for 16 hours at 37°C. On account of the disturbing effect of acid or alkali upon the ninhydrin reaction, the acidity or alkalinity of the

TABLE XI.

Effect of Acid and Alkali on the Autodigestion of Serum.

Test No.	Dialyzed guinea pig serum.	Acetone.	Acid or alkali.	Salt solution.	Concentration of reaction in medium.*	Digested in thimble. Ninhydrin test.
	cc.	cc.	cc.	cc.		
			0.1 N hydrochloric acid.			
1	2.0	1.0	1.00	0.00	N/40 hydrochloric acid.	–
2	2.0	1.0	0.50	0.50	N/80 " "	–
3	2.0	1.0	0.25	0.75	N/160 " "	–
4	2.0	1.0	0.10	0.90	N/400 " "	±
5	2.0	1.0	0.05	0.95	N/800 " "	+++
6	2.0	1.0	0.01	0.99	N/4,000 " "	+++
7	2.0	1.0	0	1.00	0	+++
			0.1 N sodium hydroxide.			
8	2.0	1.0	0.01	0.99	N/4,000 sodium hydroxide.	+++
9	2.0	1.0	0.05	0.95	N/800 " "	++
10	2.0	1.0	0.10	0.90	N/400 " "	–
11	2.0	1.0	0.25	0.75	N/160 " "	–
12	2.0	1.0	0.50	0.50	N/80 " "	–
13	2.0	1.0	1.00	0.00	N/40 " "	–

* The figures under this heading give the resulting degrees of the reaction in the mixtures. The alkalinity of the serum itself after dialysis is weaker than $\frac{N}{1,000}$ sodium hydroxide and is therefore ignored in the calculation (Test 7).

dialysates was neutralized upon the completion of digestion (Table XI). A parallel series of experiments was carried out with alanine solution as controls.

As may be seen from Table XI, the serum protease is highly sensitive to the change in the reaction of the medium. The optimal re-

action for the ferment action is that of the dialyzed serum, or at least is within the narrow limits on each side of it, either toward acid or alkaline. Even a slight deviation in the reaction beyond these limits affects the activity of the serum ferment.

Certain Chemical Reagents as Activators of the Serum Protease.

In addition to acetone, chloroform and some alcohols were found to be ferment activators, and there may be others which behave similarly. On the other hand, ethyl ether, petroleum ether, benzene, and toluene have neither an activating nor a paralyzing action. They are indifferent towards the serum protease.

Chloroform as a ferment activator has been much discussed in preceding sections. Chloroform has as much activating power as acetone. However, the simple addition of chloroform to serum does not have much effect. The mixture must be energetically and repeatedly shaken in order to insure activation. For digestion, the emulsion of the mixture as a whole may be placed in the incubator; or one may use only the upper semitransparent layer which appears when the emulsion is allowed to stand for a few minutes at room temperature, while the greater part of the clear transparent chloroform settles at the bottom of the tube. With chloroform there is no optimal proportion to be added to the serum; the ferment is not affected at all, even when the chloroform is added in excess to the serum. That chloroform requires a longer time for activating the serum ferment than does acetone has already been noted (Table VIII).

In the following experiments some monovalent saturated alcohols and ketones were tested for their activating property.

Varying amounts of different ketones and alcohols were added to 2 cc. of the dialyzed guinea pig serum in test-tubes. Before the addition of the reagents adequate amounts of salt solution were added to the serum in order that the total volume in each test should be 3 cc. With substances which are less soluble or insoluble in serum, the mixtures were repeatedly shaken. All the tubes were allowed to stand for 30 minutes at room temperature and then were transferred into dialyzing thimbles to be placed in the incubator at 37°C. for 16 hours (Tables XII and XIII).

The ketones and alcohols behave similarly towards the serum ferment. A certain optimal concentration activates ferment, and an excess injures it. Moreover, it seems to be a rule among the reagents that the higher molecular substances of the series are generally more active than the lower ones. The optimal concentration, therefore, for activating ferment was found to be approximately 33 per cent for methyl alcohol, 23 to 27 per cent for ethyl alcohol, and 20 per cent for isopropyl alcohol. This rule seems to apply also to

TABLE XII.

Activating Power of Ketones on the Serum Protease.

Dialyzed guinea pig serum.	Salt solution.	Reagent.		Test No.	Acetone.		Test No.	Methylethyl ketone.	
		Amount.	Concentration.		Appearance of mixture.	Ninhydrin test.		Appearance of mixture.	Ninhydrin test.
cc.	*cc.*	*cc.*	*per cent*						
2.0	0	1.0	$33\frac{1}{3}$	1	Turbid.	−	7	Emulsified.	−
2.0	0.3	0.7	$23\frac{1}{3}$	2	Slight turbidity.	+++	8	"	−
2.0	0.5	0.5	$16\frac{2}{3}$	3	Clear.	+	9	"	+*
2.0	0.7	0.3	10	4	"	±	10	Slight turbidity.	+
2.0	0.8	0.2	$6\frac{2}{3}$	5	"	±	11	Clear.	±
2.0	0.9	0.1	$3\frac{1}{3}$	6	"	±	12	"	±

* The optimal concentration of methylethyl ketone for digestion +++ lies between 10 and 16.7 per cent.

ketones, though the tested substances were very few. The rule is well defined only for the lower series of substances which can be mixed with water in any proportion. In the case of the higher series, which are less soluble in water, the relation is not so constant, as will be seen from the results of tests with butyl and amyl alcohols.[10] Finally, the still higher series, such as octyl alcohols, which are not soluble in water, have no activating power for the serum ferment.

[10] The isobutyl alcohol is soluble in 10.5 parts of water at 18°C., and the isoamyl alcohol in 39 parts of water at 16.5°C.

TABLE XIII.

Activating Power of Alcohols on the Serum Protease.

Reagent.		Methyl alcohol.		Ethyl alcohol.		Isopropyl alcohol.		Isobutyl alcohol.	
Amount.	Concentration.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.
cc.	*per cent*								
1.0	$33\frac{1}{3}$	Opalescent.	+++*	Strong turbidity.	−	Strong turbidity.	−	Emulsified.	−
0.7	$23\frac{1}{3}$	"	+	Opalescent.	+++	Opalescent.	+	"	−
0.5	$16\frac{2}{3}$	"	<+	"	+	"	++†	"	−
0.3	10	Clear.	±	Clear.	±	Clear.	±	Strong turbidity.	<+
0.2	$6\frac{2}{3}$	"	±	"	±	"	±	Slight turbidity.	+++
0.1	$3\frac{1}{3}$	"	±	"	±	"	±	Clear.	±
0.07	$2\frac{1}{3}$							"	±
0.05	$1\frac{2}{3}$							"	±

Reagent.		Isoamyl alcohol.		Amyl alcohol, active		Octyl alcohol, normal.		Octyl Alcohol 2.	
Amount.	Concentration.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance, of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.
cc.	*per cent*								
1.0	$33\frac{1}{3}$	Emulsified.	<+	Emulsified.	<+	Emulsified.	±	Emulsified.	±
0.7	$23\frac{1}{3}$	"	+	"	+	"	±	"	±
0.5	$16\frac{2}{3}$	"	+	"	+++	"	±	"	±
0.3	10	"	+++	"	+++	"	±	"	±
0.2	$6\frac{2}{3}$	"	+++	"	+++	"	±	"	±
0.1	$3\frac{1}{3}$	Moderate turbidity.	+++	Slight turbidity.	+++	"	±	"	±
0.07	$2\frac{1}{3}$	Slight turbidity.	±	Clear.	+	"	±	"	±
0.05	$1\frac{2}{3}$	Clear.	±	"	±	"	±	"	±
0.03	1	"	±	"	±	"	±	"	±
0.02	$\frac{2}{3}$	"	±	"	±	"	±	"	±
0.01	$\frac{1}{3}$	"	±	"	±	"	±	"	±

* The methyl alcohol, when added in a 1.5 cc. amount to 2 cc. of dialyzed serum, destroys the ferment.

† The concentration of isopropyl alcohol giving the maximal digestion is about 20 per cent.

SUMMARY AND CONCLUSIONS.

1. By means of certain chemical reagents, normal guinea pig serum can be brought to autodigestion without the presence of any foreign substrate. There exists in normal sera a highly characteristic protease.

2. The serum ferment survives heating at 55°C. for 30 minutes, but is completely inactivated at 60°C. for the same length of time.

3. The autodigestion of serum requires a temperature of about 37°C., and no noticeable digestion takes place at a temperature of 16°C. or lower.

4. Autodigestion of the serum may be brought about by chloroform and various saturated monovalent ketones and alcohols of the lower series.

5. The ketones and alcohols have a certain narrow limit of concentration for activating serum, beyond which the ferment is destroyed, even at room temperature.

6. The ketones and alcohols in concentrations regulated to activate serum at room temperature destroy the ferment when allowed to act on serum at 37°C. for 30 minutes. The elimination of the concentrated reagents from serum by evaporation or dialysis protects the ferment from their destructive action.

7. A certain length of time is required for the chemical activators to complete their action. In this respect chloroform is much slower than acetone.

8. The chemical activators may be removed from the activated serum by means of vacuum, dialysis, or extraction with certain indifferent chemicals without causing a return of the serum to its original non-autolytic state. Once activated by these reagents, the serum remains in the activated state, in spite of the removal of the activators.

9. The ferment is highly sensitive to the reaction of the medium, being readily inactivated when the reaction exceeds a certain narrow limit towards acid or alkaline. The optimal digestion is obtained with a faintly alkaline or neutral reaction.

This work was done in the laboratory of Dr. Hideyo Noguchi, under his direction.

THE AUTODIGESTION OF NORMAL SERUM THROUGH THE ACTION OF CERTAIN CHEMICAL AGENTS. II.

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It has been shown that normal serum contains a characteristic protease whose activity is revealed through the action of certain chemical activators.[1] The seroprotease shows a thermal resistance similar to that of certain proteolytic enzymes, but is peculiar in its ready destruction by the action of acetone or alcohol, to which other ferments manifest a high degree of resistance.

In the present paper we have considered the relation which this ferment bears to the various fatty and lipoidal substances and also the existence of an antiferment in serum and its relation to the seroprotease. The material and method of study have been described in the previous paper.[1]

Relation of Neutral Fats, Fatty Acids, and Lipoids to the Serum Protease.

Since all the reagents, the activating effects of which have been discussed in the previous paper, belong to the group of so called fat solvents, it is not out of place to consider what part the fat or lipoid bodies may play in the autodigestion of serum caused by these reagents. The experiments were carried out partly by adding excessive amounts of fats or lipoids to the digesting mixtures, and partly by removing the native fats and lipoids from the serum by the use of fat solvents.

Several preparations of neutral fats and lipoid bodies were dissolved in acetone in high concentration, with the exception of lecithin, which, on account of its insolubility in acetone, was dissolved in methyl alcohol. Each substance was added to 2 cc. of the dialyzed

[1] Yamakawa, S., The autodigestion of normal serum through the action of certain chemical agents. I, *J. Exp. Med.*, 1918, xxvii, 689.

guinea pig serum in two different concentrations. Some of the solutions precipitated particles of the substance when mixed with the serum and formed a layer near the surface. After standing for 30 minutes at room temperature, the contents of each test-tube were transferred into a dialyzing thimble and incubated at 37°C. for 16 hours (Table I).

That cholesterol, lecithin, and the neutral fats such as triolein and tripalmitin, even when they are added in excess to the serum, are indifferent to the process of autodigestion is proved by these experiments. The weakness of digestion in cases where fatty acids are added to the serum may be explained in various ways. As was stated in the previous paper,[1] the serum ferment is extremely sensitive to an acid reaction and is undoubtedly influenced by the fatty acids. The inhibiting power of the oleic acid was found to be much stronger than that of the palmitic acid (Tests 13, 14, 17, and 18), when they are allowed to act upon the serum ferment in equal concentration. It is not improbable that the weakness of the latter is chiefly due to its inferior solubility in a medium containing much water and to its higher melting point.

The phenomenon might be explained in another way; namely, by a specific inhibiting power of an unsaturated fatty acid such as oleic against the serum ferment. Jobling and Petersen[2] found that the unsaturated fatty acids in serum act as antitrypsin, and that they can be removed by extraction with ether or chloroform. But their results with trypsin do not find an analogy with the serum protease. As has been said, acetone or chloroform can impart their activating power to the serum ferment, and they do so without eliminating any of the native elements from it; a subsequent removal of the reagents from the activated serum does not restore the original resistance to autodigestion. Moreover, ether, toluene, benzene, and petroleum ether do not act as activators for the serum protease. The following experiment was undertaken in order to determine the effect of complete removal of the fats, fatty acids, and lipoids from the serum upon the phenomenon of autodigestion.

[2] Jobling, J. W., and Petersen, W., The nature of serum antitrypsin, *J. Exp. Med.*, 1914, xix, 459.

TABLE I.

Effect of Fatty Substances on the Autodigestion of Serum.

Test No.	Acetone solution of fats added to 2 cc. of dialyzed guinea pig serum.		Acetone.	Acid reaction. In thimble.	Acid reaction. Of dialysate.	Ninhydrin test.
		cc.	*cc.*			
1	10 per cent oleic acid.	0.8	0	+	−	<+
2		0.08	0.72	+	−	++
3	10 per cent triolein.	0.8	0	−	−	+++
4		0.08	0.72	−	−	+++
5	0.3 per cent palmitic acid.	0.8	0	<+	−	++
6		0.08	.72	<+	−	+++
7	0.3 per cent tripalmitin.	0.8	0	−	−	+++
8		0.08	0.72	−	−	+++
9	Cholesterol saturated.	0.8	0	−	−	+++
10		0.08	0.72	−	−	+++
11	Guinea pig serum alone (controls).		0.8			+++
12			0			±

Test No.	Chloroform solution of fats added to 2 cc. of dialyzed guinea pig serum.		Chloroform.	Acid reaction. In thimble.	Acid reaction. Of dialysate.	Ninhydrin test.
		cc.	*cc.*			
13	10 per cent oleic acid.	1.0	0	+	−	<+
14		0.1	0.9	+	−	++
15	10 per cent triolein.	1.0	0	−	−	+++
16		0.1	0.9	−	−	+++
17	10 per cent palmitic acid.	1.0	0	+	−	++
18		0.1	0.9	+	−	++
19	10 per cent tripalmitin.	1.0	0	−	−	+++
20		0.1	0.9	−	−	+++
21	Guinea pig serum alone (controls).		1.0			+++
22			0			±

TABLE I—*Concluded.*

Test No.	Dialyzed guinea pig serum.	1 per cent ovo-lecithin emulsion in salt solution.	Salt solution.	Methyl alcohol.	Acid reaction.		Ninhydrin test.
					In thimble.	Of dialysate.	
	cc.	*cc.*	*cc.*	*cc.*			
23	2.0	1.0		1.5	−	−	+++
24	2.0	0.1	0.9	1.5	−	−	+++
25	2.0	1.0	0	0	−	−	±
26	2.0	0	1.0	1.5	−	−	+++

10 cc. of guinea pig serum were completely dried in the desiccator by means of vacuum. The residue was ground into powder, placed in a flask, and treated with 100 cc. of absolute ether. The flask was kept for 48 hours in the refrigerator, repeatedly shaken at intervals, and the solvent three times renewed. At the expiration of this period the ether was decanted, the precipitate was washed with another 100 cc. of ether, and the trace of the solvent was removed *in vacuo*. The dried powder was then dissolved in 10 cc. of sterile distilled water and dialyzed in salt solution to remove the dialyzable substances. After dialysis the serum was diluted to 20 cc. with salt solution and used for the tests (Table II).

TABLE II.

Autodigestion of the Serum Delipolyzed with Ether.

Test No.	Extracted guinea pig serum.	Further treatment.	Ninhydrin test.
	cc.		
1	2.0	No further treatment.	±
2	2.0	Acetone 0.8 cc. added.	+++
3	2.0	Shaken with 1 cc. of chloroform.	+++
4	2.0	Methyl alcohol 1 cc. added.	+++
5	2.0	Substrate (chicken liver) added.	+++

The experiment shows that the extraction of fatty substances from the dried serum with ether causes no change with regard to the phenomenon of autodigestion of the serum.

The Inhibitory Substance in Native Serum against the Serum Protease.

It is generally known that human or animal serum has an inhibitory effect upon various proteolytic ferments, such as pepsin, trypsin, leukoprotease, and autolytic ferment. The results of the investiga-

tions on the influence of serum on the serum protease will be described here.

The investigation divided itself into two parts: (1) the digestion of heterologous substrate by the guinea pig serum ferment, and (2) the autodigestion of serum caused by the chemical reagents already mentioned. In the latter case particular care was taken to remove the chemical reagents completely from the treated serum before the sample of native serum which was to be tested for its inhibitory power was added, because, should any trace of the activators still be present, it would lead to an activation of the serum thus introduced. Acetone was used throughout the experiment because of the ease with which it can be completely removed from the serum mixtures. The dialyzed serum, acetonized and then deacetonized, will be designated, for the sake of brevity, as "activated serum."

The result shown in Table III indicates that the larger the amount of the dialyzed guinea pig serum added, the greater is the digestion of the substrate. On the other hand, the addition of a dialyzed horse serum caused neither increase nor decrease of digestion by guinea pig serum (Table IV). The horse serum itself was inactive.

The result of the autodigestion test with activated serum distinctly shows the presence of an inhibitory substance in a dialyzed but otherwise unmodified serum (Table V). The contradictory results in both cases will be discussed later.

The serum antienzymes directed against various proteolytic ferments disappear from the serum when the latter is heated to a certain temperature. The two following experiments were undertaken to determine the thermal resistance of the antiseroprotease.

1 cc. of dialyzed guinea pig serum was heated in the water bath at different temperatures for varying periods of time. The heated sera, after having been cooled, were added to 2 cc. of the activated serum in tubes, allowed to stand at room temperature for 30 minutes, and transferred as usual into thimbles for incubation and dialysis (Table VI).

According to this experiment, the inhibitory substance in unmodified or native serum withstands heating at 55°C. for 30 minutes, whereas it is destroyed by exposure at 60°C. for the same period. The thermal resistance of the antiseroprotease coincides with that

TABLE III.

Digestion of a Substrate with Guinea Pig Serum in Increasing Quantities.

Dialyzed guinea pig serum.	Salt solution.	Digested in thimble with chicken liver. Ninhydrin test.	Digested in thimble without substrate Ninhydrin test.
cc.	*cc.*		
2.0	2.0	+++	±
3.0	1.0	++++	±
4.0	0	++++	<+

TABLE IV.

Effect of a Heterologous Serum on the Digestion of a Substrate by Guinea Pig Serum.

Dialyzed guinea pig serum.	Dialyzed horse serum.	Salt solution.	Digested in thimble with chicken liver. Ninhydrin test.	Digested in thimble without substrate. Ninhydrin test.
cc.	*cc.*	*cc.*		
2.0	0	2.0	+++	±
2.0	1.0	1.0	+++	±
2.0	2.0	0	+++	±
0	2.0	2.0	±	−

TABLE V.

Inhibitory Power of the Homologous Serum against the Autodigestion of an Activated Guinea Pig Serum.

Test No.	Activated guinea pig serum.*	Homologous guinea pig serum.*	Salt solution.	Digested in thimble. Ninhydrin test.
	cc.	*cc.*	*cc.*	
1	2.0	2.0	0	<+
2	2.0	1.0	1.0	<+
3	2.0	0.5	1.5	<+
4	2.0	0.25	1.75	++
5	2.0	0.1	1.9	+++
6	2.0	0.05	1.95	+++
7	2.0	0	2.0	+++
8	0	2.0	2.0	±

* Both sera were previously dialyzed, and the mixture of both had been allowed to stand for 30 minutes at room temperature before being placed in the incubator at 37° C.

TABLE VI.

Inactivation of the Antiseroprotease of Guinea Pig Serum by Heating.

Test No.	Activated guinea pig serum.	Guinea pig serum 1 cc. exposed to various temperatures.	Ninhydrin test.
	cc.		
1	2.0	Not heated. Clear.	<+
2	2.0	30 min. at 55°C. Clear.	<+
3	2.0	30 " " 60° " Slightly turbid.	+++
4	2.0	30 " " 65° " Opalescent.	+++
5	2.0	30 " " 70° " "	+++
6	2.0	5 " " 100° " Coagulated.	+++
7	2.0	Salt solution 1 cc.	+++
8	Salt solution 2 cc.	Not heated. Clear.	±

TABLE VII.

Thermal Resistance of the Protease and Its Antisubstance in Serum.

Tests for protease.				Tests for antisubstance.				Heated serum alone.		
Test No.	Native guinea pig serum 1 cc. heated at 55°C. for.	Acetone added.	Ninhydrin test.	Test No.	Native guinea pig serum 2 cc. heated at 55° C. for.	Activated guinea pig serum added.	Ninhydrin test.	Test No.	Native guinea pig serum 2 cc. heated at 55° C. for.	Ninhydrin test.
	min.	*cc.*			*min.*	*cc.*			*min.*	
1	30	0.8	+++	6	30	2.0	<+	12	30	±
2	60	0.8	+	7	60	2.0	++	13	60	±
3	120	0.8	±	8	120	2.0	+++	14	120	±
4	240	0.8	—	9	240	2.0	+++	15	240	—
5	Guinea pig serum not heated, 2 cc.	0.8	+++	10	Guinea pig serum not heated, 1 cc.	2.0	<+	16	Guinea pig serum not heated, 2 cc.	±
				11	Salt solution 1 cc.	2.0	+++			

of the serum protease itself.[3] This fact was proved again in the next experiment.

The unmodified or native guinea pig serum, 1 cc., was exposed to a temperature of 55°C. for various periods of time. Each heated serum was mixed

[3] Yamakawa,[1] Table V.

with acetone or activated serum respectively, to be tested for its proteolytic and antiproteolytic power. After standing for 30 minutes at room temperature the mixtures were transferred into thimbles and placed in the incubator (Table VII).

Exposed to a temperature of 55°C., both the ferment and the antisubstance remain unimpaired for 30 minutes, but their activity gradually diminishes after a longer time, finally disappearing after 2 hours. A dissociation of the ferment from its antisubstance through heating was found to be impossible.

Effect of the Adsorbing Substances on the Serum.

Certain inorganic substances, which had been previously sterilized by heating, were put into the dialyzed guinea pig serum in a proportion of 5 gm. to 10 cc. The mixtures were allowed to stand at room

TABLE VIII.

Digesting Power of the Serum Treated with Adsorbents.

Test No.	Kind and amount of guinea pig serum.	Further treatment.	Ninhydrin test.
1	Serum treated with kaolin 2 cc.	No further treatment.	−
2		Acetone 0.8 cc. added.	−
3		Substrate added.	−
4	Serum treated with charcoal 2 cc.	No further treatment.	−
5		Acetone 0.8 cc. added.	±
6		Substrate added.	−
7	Serum treated with talc 2 cc.	No further treatment.	−
8		Acetone 0.8 cc. added.	±
9		Substrate added.	−
10	Serum treated with silicious marl 2 cc.	No further treatment.	−
11		Acetone 0.8 cc. added.	±
12		Substrate added.	−
13	Serum treated with barium sulfate 2 cc.	No further treatment.	−
14		Acetone 0.8 cc. added.	±
15		Substrate added.	−
16	Untreated serum 2 cc. (controls).	No further treatment.	±
17		Acetone 0.8 cc. added.	+++
18		Substrate added.	+++

temperature for an hour, with repeated shakings, and then centrifuged. The clear supernatant fluids were used for the experiment (Table VIII).

As may be seen from Tables VIII and IX, the proteolytic ferment can be easily removed from serum by adsorbents, but the antisubstance, on the other hand, still remains in the treated serum.

TABLE IX.

Antienzymic Action of the Serum Treated with Adsorbents.

Test No.	Kind and amount of guinea pig serum.		Activated serum added.	Acetone added.	Ninhydrin test.
		cc.	*cc.*	*cc.*	
1	Guinea pig serum treated with kaolin.	2.0	0	0	−
2		2.0	0	0.8	−
3		1.0	2.0	0	<+
4	Guinea pig serum treated with talc.	2.0	0	0	−
5		2.0	0	0.8	±
6		1.0	2.0	0	<+
7	Salt solution.	1.0	2.0	0	+++
8	Guinea pig serum.	1.0	2.0	0	<+

Occurrence of the Proteolytic Ferment and Its Antisubstance in the Sera of Different Animals.

It would surely have been an advantage if we could have found larger animals which would furnish us with a serum as rich in the

TABLE X.

The Proteolytic Ferment and Its Antisubstance in the Sera of Different Animals.

Kind of serum.	No. of tested specimens.	Dialyzed serum alone 2 cc.	Dialyzed serum 2 cc. + acetone 0.8 cc.	Dialyzed serum 1 cc. + activated guinea pig serum 2 cc.
Human serum	2	+	<+	<+
Dog "	5	±	<+	<+
Cat "	2	±	<+	<+
Rabbit "	8	±	<+	<+
Horse "	2	−	<+	<+
Guinea pig serum	Over 100	±	+++	<+

serum protease as that of the guinea pig. The results of examinations of various animal sera, however, showed that the guinea pig is the only animal whose serum is exceedingly rich in the proteolytic ferment. On the other hand, the sera of other animals, while poor in their content of protease, contain a considerable amount of the antisubstance capable of counteracting the action of the autolytic ferment of guinea pig serum. The result of the digestion tests with the sera of different animals is shown in Table X.

Mode of Digestive Action of the Serum Ferment.

It has been stated in a previous paragraph that the proteolytic ferment of serum, when it is incubated with substrate, can produce the dialyzable substances despite the presence of native serum, while in the autodigestion of activated serum, the ferment action is inhibited by the addition of native serum. There seems to be a certain difference in the mode of action in the two instances.

The explanation of the autodigestion of the activated serum may probably be sought in the destruction or paralysis of the antienzymic substance through the treatment. Reagents such as certain ketones and alcohols, when their optimal concentration for activation is reached, may destroy the antienzyme, but not the enzyme, thus enabling the latter to exert its full activity upon the serum proteins. The concentration of reagent which dissociates the ferment from its antisubstance lies between narrow limits, and when it exceeds the upper limit, the ferment itself is also destroyed.

In autodigestion the protein in the treated serum must serve as substrate, because there is nothing else present to be hydrolyzed. But what is the origin, then, of the dialyzable substance produced when the serum is incubated with various tissue substrates? There are two possibilities for the source of the protein derivatives: first, the substrates may be directly digested by the serum ferment; second, it may be assumed that the homologous tissues are not really digested, but that they act only as an adsorbing agent which removes the antienzyme and leaves the freed autolytic ferment to digest its own serum protein. The latter explanation was advanced by Bron-

fenbrenner[4] in the Abderhalden reaction, in which pregnant human serum, when incubated with placenta tissue, gives a positive ninhydrin test. He states that pregnant serum is able to show autodigestion in the incubator when allowed to remain in contact with

TABLE XI.

Antienzymic Action of Normal Serum after Treatment with Substrate at 0.5°C.

Test No.	Dialyzed guinea pig serum.	Further treatment.			Digested in thimble. Ninhydrin test.
	cc.				
1	2.0	Substrate added. Tubes left at 0.5° C. for 16 hrs.	Centrifuged. Substrate removed. Supernatant fluid alone used for tests.		±
2	2.0			Boiled.	−
3	2.0			Acetone 0.8 cc.	+++
4	1.0			Activated guinea pig serum 2 cc.	<+
5	2.0		Substrate *in situ*.		+++
		Control tests.			
6	2.0	Without any treatment.			±
7	2.0	Acetone 0.8 cc. added.			+++
8	2.0	Substrate added.			+++
9	1.0	Activated guinea pig serum 2 cc. added.			<+
10	Salt solution 1 cc.	Activated guinea pig serum 2 cc. added.			+++

placenta tissue for 16 hours on ice and then separated from the substrate. He ascribes the phenomenon to adsorption of the antienzymic substance by the substrate impregnated with a specific antibody contained in the serum of a pregnant subject.

The next experiment was undertaken to determine whether this

[4] Bronfenbrenner, J., On the present status of the Abderhalden reaction, *J. Lab. and Clin. Med.*, 1915–16, i, 79.

mode of interpretation was applicable in our case, in which a heterologous, non-specific substrate is treated with normal serum (Table XI).

Tests 1 and 4 show that normal guinea pig serum, when it is kept on ice with substrate, is neither activated nor deprived of its antienzymic substance. In other words, the normal serum is indifferent to the treatment, contrary to the result which Bronfenbrenner reported to have obtained with human pregnant serum and placenta tissue. But this result does not exclude the possibility of the adsorption of the antisubstance by ordinary substrates in the incubator at a temperature of 37°C.

To determine the fate of the antienzymic substance in serum, after digestion, the serum was treated according to four methods, as follows:

Serum A.—8 cc. of the dialyzed guinea pig serum were kept in the incubator with substrate in four thimbles for 16 hours, 2 cc. being placed in each thimble. At the expiration of this time, when the dialysate showed a ninhydrin reaction of +++, the serum in the thimbles was separated from the substrate layer and every trace of the latter removed by means of centrifugation. The serum was then dialyzed in a celloidin sac against salt solution to eliminate the split products of protein contained in it.

Serum B.—8 cc. of the activated guinea pig serum were kept in thimbles in the incubator for 16 hours. The ninhydrin test of the dialysate showed a reaction of +++. The sera in the thimbles were reunited and dialyzed as mentioned above.

Serum C (Control 1).—The dialyzed guinea pig serum, 8 cc., without any substrate and without being activated, was treated in the same way as the other two sets of serum; *i.e.*, incubated in thimbles and afterwards dialyzed.

Serum D (Control 2).—The dialyzed guinea pig serum without any preliminary treatment.

These sera were further treated as shown in Table XII and digested in thimbles at 37°C. for 16 hours. The dialysates were tested as usual.

The result of this experiment indicates that the serum, the proteolytic power of which has already been exhausted by treatment with substrate, still contains its antienzymic substance (Serum A, Test 4), while the latter is no longer found in the activated serum after digestion (Serum B, Test 4). There is no doubt that in the former case the digestion can take place in spite of the presence of

the antienzymic substance. We have no more reason in this instance to assume the occurrence of an indirect digestion of serum protein, due to the absorption of the antienzymic substance through the substrate, because the substrate does not absorb the antienzyme under the experimental conditions here recorded. It seems justifiable, therefore, to conclude that the serum ferment directly digests the protein of the heterologous substrate, while in the case of the activated serum, the ferment splits its own serum protein after the antienzymic substance has been removed by the treatment with ketones or alcohols.

TABLE XII.

Fate of the Antienzyme Substance in Serum after Digestion.

Test No.	Amount of serum.*	Further treatment.	Serum A	Serum B	Serum C	Serum D
	cc.					
1	2.0	No further treatment.	−	−	−	±
2	2.0	Acetone 0.8 cc.	−	±	++	+++
3	2.0	Substrate.	−	+	++	+++
4	1.0	Activated guinea pig serum 2 cc.	<+	+++	<+	<+
5	1.0	Dialyzed guinea pig serum 2 cc. + substrate.	+++	+++	+++	+++

* The volume of the serum was increased after secondary dialysis by about one-fourth.

SUMMARY AND CONCLUSIONS.

1. The neutral fats, fatty acids, and lipoid bodies of serum seem to play no part in autodigestion. Neither the addition of fats or lipoids in excess to the serum, nor their removal by extraction with ether influences the phenomenon of autodigestion.

2. There is present in native serum an antienzymic substance which is closely related to the autolytic ferment of serum.

3. The antiseroprotease of normal serum has almost the same thermal resistance as the seroprotease; that is, it survives heating at 55°C. for 30 minutes but is completely inactivated at 60°C. for the same length of time.

4. The ferment can be removed from the serum by means of inorganic adsorbents, but the antienzymic substance remains in the treated serum.

5. The autolytic power of the sera of man and other animals is much weaker than that of guinea pig serum, but they contain as much as does the latter of the antisubstance which inhibits the digestion of the activated guinea pig serum.

6. The autodigestion of the activated serum is due to the splitting of the serum protein by the proteolytic ferment of the same serum and is brought about by the destruction of the antienzymic substance by the chemical reagents. On the other hand, the digestion products in a mixture of a foreign substrate and guinea pig serum are derived from the direct digestion of the substrate by the serum ferment. This digestion takes place in spite of the presence of the antiseroprotease. The serum separated from the substrate can no longer produce a split product, but is as actively antienzymic as the original serum and undergoes autodigestion only when treated with acetone or other chemical activators.

This work was done in the laboratory of Dr. Hideyo Noguchi, under his direction.

ANTIBODY PRODUCTION AFTER PARTIAL ADRENALECTOMY IN GUINEA PIGS.

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Aside from the well established action of products of adrenal secretion upon sympathetic nerve endings and their consequent effect upon striated and smooth musculature, there has been assigned to the adrenal glands a detoxifying action upon endogenous and exogenous poisons which would class them among the specific defensive mechanisms of the body. Studies of this action have been limited largely to observations and experiments to show the effects of acute and chronic intoxications upon the adrenal glands, the symptomatology of adrenal hyperplasia or degeneration, the effect of partial or complete extirpation upon the toxicity of various substances, or the neutralization of toxins by adrenal extracts in the test-tube or the living animal. The literature is confused and contradictory, and much of the evidence brought forward is speculative and indirect, due in part to the use of the death or survival of the animal as sole indicator, and the consequent introduction of many unknown factors into the experimental equation, without critical analysis of the mechanism involved.

Only a few experiments have come to light which bear directly upon a possible relation between the adrenal glands and the recognized immunity factors of the defensive mechanism, and these reported findings are almost entirely of a negative significance. Thus Josué and Paillard[1] found that injections of adrenalin or of adrenal extracts into rabbits had no effect on the opsonic properties of the blood. Hektoen and Curtis[2] report that "Adrenalectomy in normal dogs, and in dogs at the height of the antibody curve after the injection of rat blood, did not cause any fall in the antibody content of the blood serum, as determined by hourly

[1] Josué, O., and Paillard, H., Influence de l'adrénalin sur le pouvoir opsonique (Première note), *Compt. rend. Soc. biol.*, 1910, lxviii, 657.

[2] Hektoen, L., and Curtis, A. R., The effect on antibody production of the removal of various organs, *J. Infect. Dis.*, 1915, xvii, 409.

observations after the operation and until death." Gay and Rusk[3] in presenting studies on antibody formation, in which the literature is reviewed, make no mention of the adrenals as possible sources of immune substances, and all the positive evidence reported in regard to the origin of immune bodies points to other organs, notably the lymph glands, bone marrow, and spleen, as the tissues probably concerned. A solitary communication by Cattoretti[4] states that the addition of pancreas extract to the blood of adrenalectomized rats gave a marked lowering of the surface tension (miostagmin reaction) compared with the normal lowering produced by the extract.

There is no confirmed evidence that the adrenal glands play an active part in antibody formation, or in the known immunity reactions of defense against bacterial invasion. On the other hand, the few experiments which have been made to study such a relation have given negative results.

As a part of a more general investigation of the possible relation of glands of internal secretion to immunity processes we have made experiments on the effect of partial adrenalectomy upon antibody formation in guinea pigs. For this purpose the animals were subjected to operation before or after immunization with a typhoid vaccine or with washed red blood corpuscles of the hen, and their typhoid agglutinins or hemolysins and hemagglutinins titered at intervals during the course of antibody production.

Healthy adult guinea pigs, usually males, weighing from 300 to 400 gm., served as the experimental animal. For the purposes of the experiments it was necessary that the animals should survive during the interval required for subsequent active immunization or for the change in antibodies already in circulation, in the event that adrenalectomy modified the response. Consequently, complete removal of the glands was interdicted, since guinea pigs are practically without accessory adrenal tissue and almost invariably die within a few hours of a total extirpation.[5]

Operations for the partial removal of the adrenals were performed

[3] Gay, F. P., and Rusk, G. Y., Studies on the locus of antibody formation, *Tr. XVth Internat. Cong. Hyg. and Demog., Washington (1912)*, 1913, ii, 328.

[4] Cattoretti, F., Ueber die Meiostagminreaktion bei den weissen Ratten nach Extirpation der beiden Nebennieren, *Wien. klin. Woch.*, 1911, xxiv, 637.

[5] Lucien, M., and Parisot, J.-V.-J., Glandes surrénales et organes chromaffines, Paris, 1913, 108.

with careful aseptic technique, and the endeavor to reduce shock to the minimum by prompt hemostasis, the use of a warm pad during and after operation, machine-controlled anesthesia with constant ether concentration, and saline injections when indicated. We deemed it essential carefully to avoid complications due to infection or to injury to other organs in the course of the operation.

Operation.—Oblique incisions on both sides, separating the last two ribs, and extending to the edge of the sacrospinal muscles gave a clear exposure of the glands with the least disturbance of the abdominal contents. The adrenals were dissected free to the hilum, caught in a delicate curved mosquito clamp at the base or through their substance, and cut free distal to the clamp. Only slight bleeding resulted if the clamp was left in place a short time. The glands or portions of glands removed were weighed to estimate the amount taken and sectioned for comparison post mortem with the segment left in place.

It was soon found that the guinea pig could stand the loss of the whole of one gland and from one-half to three-fourths of the other. If too much tissue was taken the animal died in from a few hours to several days, after showing characteristic symptoms. A marked fall in temperature (to 28°C. in one instance), extreme prostration, gasping respiration, intermittent clonic convulsions, and, in males, the extrusion of semen immediately preceded death. These findings are in accord with former reports.

On the other hand, surviving guinea pigs recovered quickly from the operation and remained well for months, sometimes losing at first 50 to 100 gm. in weight, which was often recovered later. Some of the animals died during the course of the experiments, however, from hemopericardium after cardiac puncture or from an intercurrent lung epizootic prevalent among the stock.

Technique of the Serum Reactions.—The strain of *B. typhosus* chosen was a stock culture known as "Sen," recovered by Dr. Bull from an ampule of Besredka's sensitized vaccine. It had been on artificial media for several years and combined ready agglutinability with a high toxicity for guinea pigs, often killing them in the usual immunizing doses. For this reason and to obtain exact dosage, a vaccine was prepared by suspending 24 hour growths from Blake bottles in saline solution, killing the bacilli with chloroform, disrupting them by repeated freezing and thawing, dehydrating *in vacuo* over sulfuric acid, and powdering in a mortar. Weighed quantities were resuspended in normal saline solution, extracted by shaking for several hours, and measured doses corresponding to 0.5

to 2 mg. of the powder were injected intraperitoneally at 3 to 4 day intervals for three doses. Initial agglutinin titers ranged from 1: 640 to 1: 5,120 on the 7th to 10th day after the final injection. Ten of twenty-four guinea pigs gave an initial titer of 1: 1,280. In order to simplify the experiments only the typhoid agglutinins were followed. Casual tests showed that this method of immunization, while simple and rapid, did not produce precipitins or complement-fixing bodies in concentrations suitable for investigation.

Other guinea pigs were immunized with red blood corpuscles of barred Plymouth Rock chickens. Following Coca's[6] schedule three intraperitoneal doses of 0.5 to 1.5 cc. of washed corpuscles, made up to the original blood volume, were injected at 4 day intervals and the guinea pigs were first bled a week later. The first hemagglutinin titers ranged from 1: 160 to 1: 1,280, hemolysins appearing in dilutions five times as great, taking into account the dilutions involved in the hemolytic system. For the agglutination tests one drop of a suspension of *B. typhosus*, Sen, or of washed hen corpuscles (10 per cent of the original blood volume), was added to a 1 cc. volume of successive dilutions of the fresh, inactivated guinea pig serum. The tubes were read first after 2 hours at 37°C. and 2 hours at room temperature. A confirmatory reading was taken after the tubes had stood in the ice box over night. To test hemolysins, 0.25 cc. volumes of inactivated experimental serum, fresh guinea pig complement 1: 10, and 5 per cent hen corpuscle suspension were made up with 0.5 cc. of saline solution, following the usual technique of the Wassermann reaction. These tubes were incubated 1½ hours, being shaken at the half hour and hour, and read immediately, and after standing in the ice box over night. All the tests were performed with the usual controls.

With these methods several series of experiments were performed. Usually three guinea pigs formed the experimental unit. In some instances two were partially adrenalectomized, the other serving as a normal control. In other cases two animals served as controls, one normal, the other after an operation similar to double adrenalectomy except for the removal of the glands. The control operated animals showed that the operation itself had no influence on antibody formation.

Series I.

In some of the experiments in this series the guinea pigs were first immunized with *Bacillus typhosus* vaccine and their agglutinin titers recorded on the 7th day after the third injection. Within a few days

[6] Coca, A. F., A rapid and efficient method of producing hemolytic amboceptor against sheep corpuscles, *J. Infect. Dis.*, 1915, xvii, 361.

(8 to 11 days after immunization) the adrenalectomies or control operations were performed and the agglutinin titers followed in 2 cc. samples of blood obtained by cardiac puncture at intervals during the succeeding weeks and months. In other experiments the operations preceded immunization, which was begun from 1 to 52 days later. In a few instances one gland or a part of a gland was removed, the

TABLE I.

Differences in Agglutinin Titer of Various Sera on Reexamination after an Interval of Time.

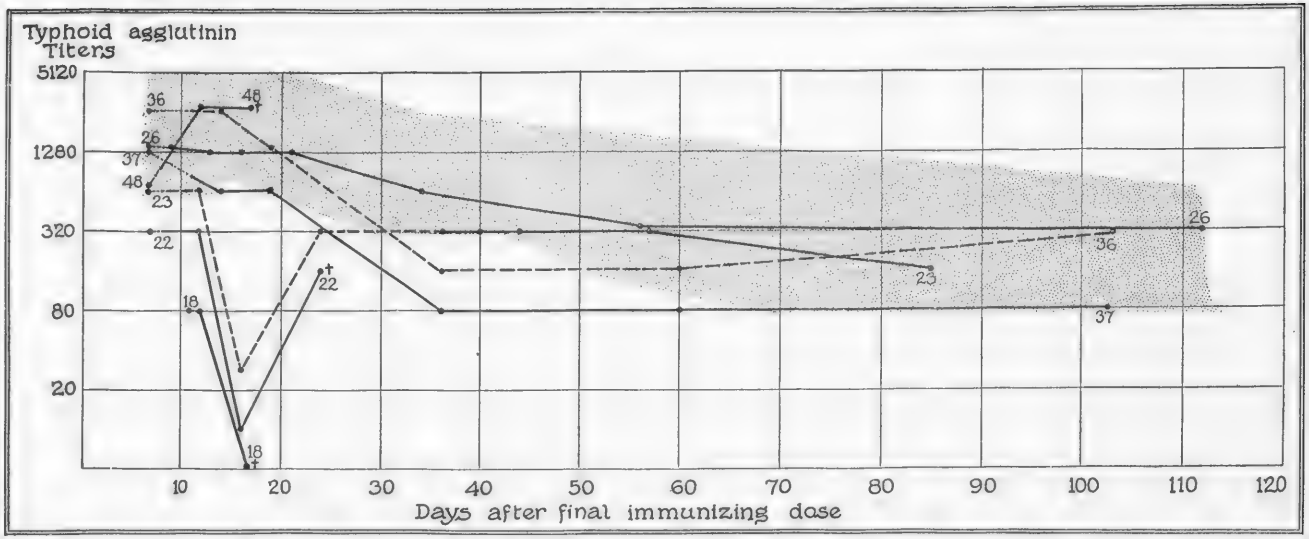
Animal No.	Condition.	Serum of.	Interval between tests.	First titer.	Second titer.
			days		
22	Double adrenalectomy almost complete.	Dec. 27, 1916	35	1:30	1:640
		Jan. 4, 1917	26	1:320	1:1,280
23	Right adrenal removed.	Dec. 27, 1916	35	1:160	1:640
		Jan. 4, 1917	26	1:320	1:320
	Three-fourths of left adrenal removed.	Jan. 16, 1917	15	1:320	1:640
		" 20, 1917	7	1:320	1:320
		Mar. 5, 1917	7	1:160	1:320
24	Normal.	Dec. 27, 1916	35	1:1,280	1:2,560
		Jan. 4, 1917	26	1:1,280	1:2,560
	Control operation.	Jan. 16, 1917	15	1:1,280	1:1,280
		" 20, 1917	7	1:640	1:1,280
		Mar. 5, 1917	7	1:160	1:320
2	Double adrenalectomy.	Mar. 5, 1917	7	1:160	1:640
		" 12, 1917	14	1:640	1:640
39	Normal.	Mar. 5, 1917	7	1:160	1:160

guinea pig immunized, and the second gland or part of it taken at a later date. These two sets of experiments, in which the effect of partial adrenalectomy was studied in animals previously or subsequently immunized to *Bacillus typhosus*, form a group in which all the control animals may serve as a basis for comparison with those on which the extirpation of adrenal tissue was performed. The agglutinin curves of the control animals showed considerable variations

due to individual reaction, so that a plot of all the normal curves gives a confusion of lines within rather wide limits on the chart. Obviously comparison of the antibody curve of an adrenalectomized animal with its own control alone might indicate differences in reaction not due essentially to the loss of adrenal tissue. Therefore it seems best simply to outline an area which covers all the variations in reaction found in normal guinea pigs. Against this normal area the separate curves of agglutinin formation in the experimental animals may be charted. Following this method Text-fig. 1 shows the curves of seven guinea pigs which were adrenalectomized subsequent to immunization. It will be seen that the titers of all but three of them fall practically within the limits of normal variation. The findings in the exceptional cases require special comment.

Immediately after operation the agglutinin titers of the sera of these animals appeared to drop sharply almost to zero, with as sharp a rise later in the two guinea pigs which survived. No similar drop was apparent in the titer of the control. These readings were made on the fresh inactivated sera the same day they were taken. When observations on other animals failed to confirm this finding, these sera, which had been kept in the dark at ice box temperature for about a month, were reexamined, and now gave titers more nearly corresponding to those of the later experiments. The differences in titer are shown in Table I, with the record of subsequent analyses in which sera were studied while fresh, and again after standing several days. In a number of instances it will be seen that they agglutinated the Sen strain in higher dilutions on reexamination. Since this observation was made on normal as well as adrenalectomized guinea pigs the difference must be sought in some other factor, which would account for the apparent inhibition in fresh sera. Moreover, this initial inhibition does not appear with regularity. For uniformity it is necessary to use the agglutination titers from the fresh sera in drawing conclusions from the experiments, but it is apparent that disturbing factors are latent in the results.

With the exceptions noted above, these experiments indicate that adrenalectomy subsequent to immunization has no significant effect upon the curve of typhoid agglutinin formation in guinea pigs. Similar results were found in the experiments charted in Text-fig. 2, in which, against the normal background are seen the agglutination curves of seven guinea pigs which were immunized subsequent to adrenalectomy. Their reactions to immunization fall substantially within normal limits.



..... Period before operation.
—— —— —— —— One adrenal removed.
———————— One, and most of the other adrenal removed.
The shaded area covers the variations in titers of the controls.

TEXT-FIG. 1. Typhoid agglutinin titers of animals immunized before adrenalectomy.

Partial adrenalectomy, with removal of a single gland, or of one gland and as much of the other as can be taken with impunity, appears to have no influence upon the formation of typhoid agglutinins in guinea pigs.

Series II.

Cole[7] has described a late effect of immunization which persists after demonstrable immune bodies have disappeared from the blood.

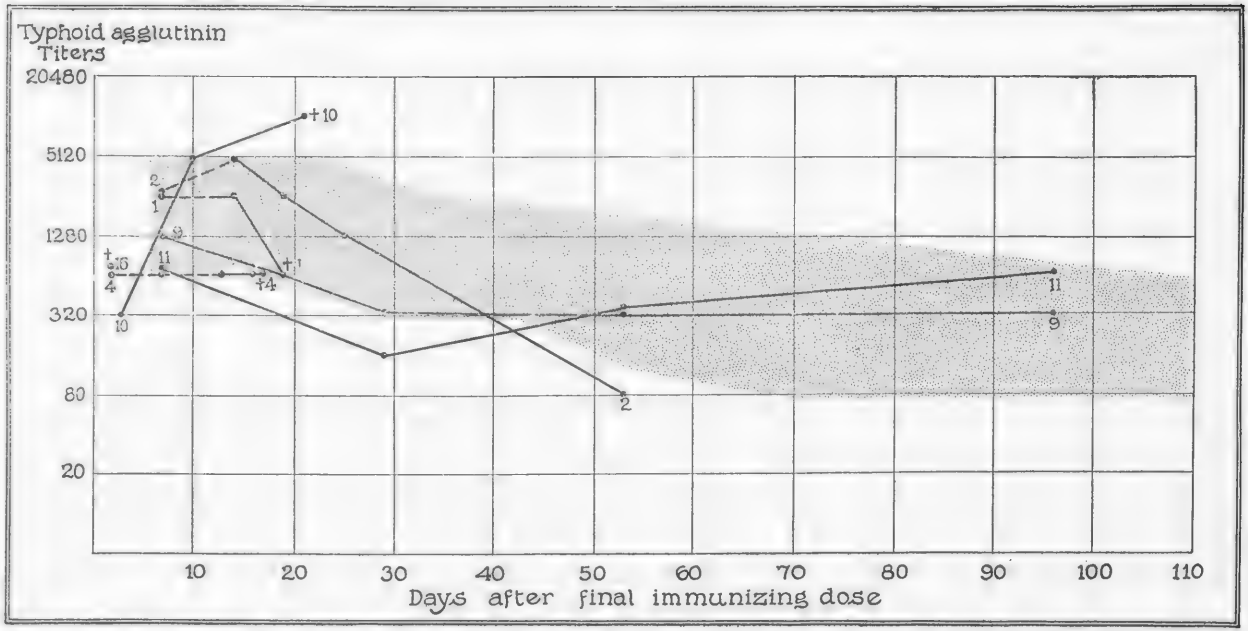
TABLE II.

Rise in Agglutinin Titers of Adrenalectomized and Control Guinea Pigs on Reinjection of Typhoid Vaccine.

Animal No.	Previous history.	Serum agglutinin titer on Mar. 5, 1917.		Serum agglutinin titer on Mar. 12, 1917.	Serum agglutinin titer on Apr. 24, 1917.
23	Adrenalectomized; immunized 84 days before.	1:160		1:320	1:80
24	Operated control; immunized 84 days before.	1:160	Mar. 5, 1917. Injected intraperitoneally 0.05 mg. of typhoid vaccine.	1:640	1:160
2	Immunized 53 days before; then adrenalectomized.	1:80		1:640	1:320
39	Normal; immunized 53 days before.	1:160		1:320	1:160
60	Normal.	0		1:20	1:20
61	"	0		0	1:10

Reinjection of previously immunized animals with a minute dose of the original antigen, an amount which has no effect upon normal animals, causes a sharp rise in the antibody curve. This effect is interpreted as due to a latent tissue sensitization. To test the presence of this phenomenon in adrenalectomized animals, two of the survivors from the experiments already described, with their controls, and with two normal guinea pigs, were injected intraperitoneally with 0.05 mg. of the typhoid vaccine used for the original immunization. At this time the animals from the earlier experiments

[7] Cole, R. I., Experimenteller Beitrag zur Typhusimmunität, *Z. Hyg. u. Infectionskrankh.*, 1904, xlvi, 371.

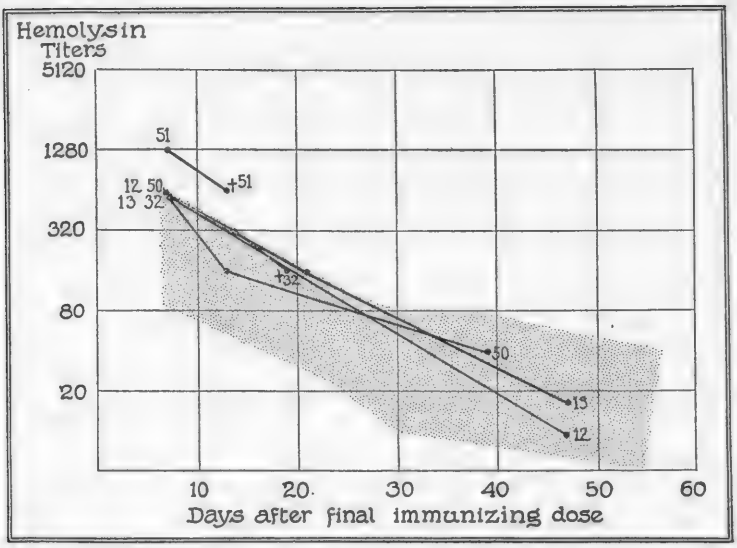


TEXT-FIG. 2. Typhoid agglutinin titers of animals immunized after adrenalectomy.

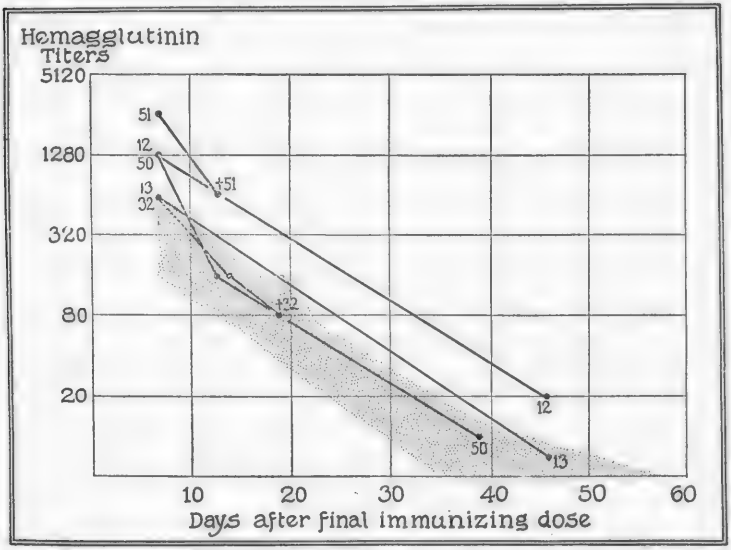
showed typhoid agglutinins in dilutions of 1:80 to 1:160 of their sera. The normal controls showed no antibodies. 7 days later the titers of the previously immunized guinea pigs had risen to 1:320 to 1:640, while the single dose of 0.05 mg. of vaccine had resulted in agglutinin formation to the extent of 1:20 in one control. These findings, and the results of a subsequent titration are found in Table II. The tissue sensitization occurred normally in the partially adrenalectomized guinea pigs and their previously immunized controls.

Series III.

With the technique already described a smaller number of experiments was made on guinea pigs immunized against hen corpuscles. Hemagglutinins and hemolysins were studied in animals adrenalectomized before or after immunization in experiments similar to those in Series I. Although the initial antibody titers corresponded roughly to those obtained with typhoid vaccine, the hemagglutinins and hemolysins disappeared from the circulating blood much more rapidly, so that the antibody content was reduced almost to nothing in the course of 2 months. The results of titrations at intervals are shown in Text-figs. 3 and 4, in which, against a background of normal variations are plotted the curves of the adrenalectomized animals. Two of these, namely Guinea Pigs 12 and 13, were adrenalectomized before immunization, losing one-half the right gland 43 days, and the left gland 7 days before the first injection of corpuscles. The other adrenalectomized animals, namely Guinea Pigs 32, 50, and 51, were first immunized and then operated upon 7 to 10 days after the final injection. The high initial agglutinin titers of Guinea Pigs 50 and 51 are due to four, instead of the usual three injections. Of the three animals in this experiment, those with the highest titers were chosen for operation. The control animal, Guinea Pig 52, had an initial hemagglutinin titer of 1:640. The slopes of the curves for Guinea Pigs 50 and 51 are seen to parallel the normal curve, although the titers for Guinea Pigs 50 and 51 do not fall within the normal limits. The charted results show that adrenalectomy has no essential influence upon the production or gradual diminution of hemolysins and hemagglutinins in guinea pigs.



TEXT-FIG. 3. Hemolysin titers of adrenalectomized animals. Guinea Pigs 12 and 13 were adrenalectomized before immunization and Guinea Pigs 32, 50, and 51 after immunization.



TEXT-FIG. 4. Hemagglutinin titers of adrenalectomized animals. Guinea Pigs 12 and 13 were adrenalectomized before immunization and Guinea Pigs 32, 50, and 51 after immunization.

Series IV.

As with the survivors from the experiments with typhoid agglutinins, the guinea pigs immunized against hen corpuscles were given a second small injection of the foreign blood cells after antibodies had almost disappeared from their sera. With two normal controls, and

TABLE III.

Rise in Hemolysin and Hemagglutinin Titers of Previously Immunized Guinea Pigs on Reinjection with Hen Corpuscles.

Animal No.	Previous operative history.	Previous immunization.	Hemolysins.			Hemagglutinins.		
			Time before injection.	Time after injection.		Time before injection.	Time after injection.	
			5 days.	7 days.	45 days.	5 days.	7 days.	45 days.
13	Double adrenalectomy 68 days before.	52 days before.	1:10	1:160	1:40	0	1:20	1:20
31	Double adrenalectomy 4 days before.	61 " "	0	1:320	1:20	0	1:40	1:10
43	Double adrenalectomy 4 days before.	52 " "	1:10	1:320	1:20	0	1:80	1:20
50	Double adrenalectomy 37 days before.	44 " "	1:40	1:320	1:40	1:10	1:40	1:40
55	Double adrenalectomy 3 days before.	40 " "	1:40	1:160	1:40	0	1:40	1:20
54	Right adrenal removed 3 days before.	40 " "	1:10	1:320		0	1:40	
33	Control operation 51 days before.	61 " "	1:40	1:160	1:40	0	1:40	1:20
44	None.	52 " "	1:10	1:160	1:40	0	1:40	0
52	"	44 " "	1:40	1:320	1:80	1:10	1:40	1:10
53	"	40 " "	1:20	1:20	1:10	0	1:20	1:10
62	None; normal.	None.	0	0	0	1:80	1:40	1:10
63	" "	"	0	0	0	0	0	0

four control guinea pigs previously immunized, six partially adrenalectomized animals were injected intraperitoneally with 0.05 cc. of washed hen corpuscles. One of the adrenalectomized guinea pigs had been operated upon before the previous immunization. The other four had lost adrenal tissue at varying intervals after immunization. Although the minute dose of antigen given did not stimu-

late antibody production to the original level, the adrenalectomized and all but one of the control animals which had been previously immunized responded with hemagglutinin titers of 1: 20 to 1: 80, and hemolysin titers of 1: 160 to 1: 320 on the 7th day, whereas the normal control animals, for which this was the first injection, failed to show demonstrable increase of antibodies. Control Guinea Pig 62 with an initial hemagglutinin titer of 1: 80 before injection gave subsequent titers of 1: 40 and 1: 10, showing no effect from the injection. 5 weeks later the hemolysins of most of the animals had fallen below 1: 80, and the hemagglutinins below 1: 40. These results are given in Table III.

In addition to the experiments described above on guinea pigs, three rabbits were partially adrenalectomized and tested for *in vivo* agglutinins, as described by Bull.[8] Typhoid bacilli injected on the 4th or the 24th day after adrenalectomy were clumped and removed from the blood stream with the same rapidity and completeness as in normal animals. The blood of these rabbits was then tested for natural typhoid agglutinins by the usual method *in vitro*. The titers ranged from 1: 16 to 1: 128, showing that neither adrenalectomy nor the *in vivo* agglutination had removed these antibodies from the blood.

SUMMARY.

By careful aseptic operation it was found possible to remove approximately three-quarters to seven-eighths of the adrenal tissue of guinea pigs without causing symptoms of adrenal insufficiency. Guinea pigs were immunized to *Bacillus typhosus* or to hen corpuscles at varying intervals before or after the operation, and the curves of antibody formation traced for 2 to 3 months after immunization. Comparisons with the antibody curves of control animals similarly immunized fail to show that the adrenalectomy had any influence upon the rise or persistence of antibodies in the blood.

For the purposes of the study it was not deemed necessary to produce an acute adrenal insufficiency. If the adrenal glands were the site of antibody formation or played an essential part in immunity processes, it does not seem probable that the small remainder of

[8] Bull, C. G., The agglutination of bacteria *in vivo*, *J. Exp. Med.*, 1915, xxii, 484.

adrenal tissue left *in situ* to sustain life would affect quantitatively the antibody response to a given antigen injection as do the entire normal glands. We therefore interpret the experiments to indicate that not only are the adrenal glands not one of the important sources of typhoid agglutinins, or of hemagglutinins or hemolysins, but they play no essential part in the mechanism by which these antibodies are produced and maintained in the body.

ÆSTIVO-AUTUMNAL MALARIA. THE EXTRACELLULAR RELATION OF THE CRESCENTIC BODIES TO THE RED CORPUSCLE AND THEIR METHOD OF SECURING ATTACHMENT.*

By MARY R. LAWSON, M.D.

(*From the Laboratory of Dr. Mary R. Lawson, New London.*)

Plates 33 to 35.

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Æstivo-Autumnal Parasite.

Æstivo-autumnal parasites are extracellular throughout their life cycle; they migrate from one red corpuscle to another, destroying several in the course of their development. They pass through a sexual cycle in the human host,[1] with the formation of flagella by the microgametocyte, fertilization of the macrogamete, and its subsequent segmentation. I have seen these phases many times. The great difficulty in working out the phases in the æstivo-autumnal infections is that since the infection is usually so serious, one hardly feels justified in withholding treatment of the infected individual for the period of time necessary for the parasites to become very numerous, or, if already numerous, for the time required for a protracted study of them during their developmental phases. (The developmental and sexual phases rarely appear in the peripheral blood unless the patient has a very heavy infection.) It requires many hours for the complete examination of even one film of the patient's blood.

Crescentic Bodies.

The life phase of the æstivo-autumnal parasite is represented by the characteristic crescentic bodies. They develop in gradual stages

* Aided by a grant from The Rockefeller Institute for Medical Research.

[1] Rowley-Lawson, M., The æstivo-autumnal parasite: its sexual cycle in the circulating blood of man, with a description of the morphological and biological characteristics of the parasite, *J. Exp. Med.*, 1911, xiii, 263.

from the small ring-form parasites as round bodies, finally opening out into the crescent form. Several days are required for their development, which usually takes place in the internal organs of the host. The crescent may assume other forms, such as fusiform, ovoid, and round. Many of the so called round and ovoid bodies are (*a*) crescents viewed from their convex side; (*b*) crescents bent on themselves; (*c*) adult crescents contracted into round bodies and ovoids. The contracted round bodies and ovoids are usually seen at the edges of films made on slides and cover-slips. By the examination of many specimens one soon learns to distinguish between the developing (round body) crescent and the round form assumed by the adult crescent.

a b

TEXT-FIG. 1, *a* and *b*. The appearances one would get if the parasite were within the corpuscle; that is, the outline of the decolorized corpuscle would correspond to the convexity of the crescentic body. One would not expect it to embrace the ends of the crescent, and then bulge out from the concavity of the crescent in the curved line, as shown in Text-fig. 2, *a*, *b*, and *c*. It would be more likely to present an appearance such as is shown in Text-fig. 1, *b*.

Extracellular Relation of the Crescent to the Red Corpuscle.—The crescentic bodies are generally believed to be within the substance of the red corpuscle in spite of the many evidences, easily demonstrated, that they are attached to its external surface. Even if one could not understand the method by which this attachment was secured, the relation of the "bib" to the parasite should enable one to see that they are not within the corpuscle. For instance, if they were within the corpuscle, one would expect to get such appearances as are shown in Text-fig. 1. As a matter of fact, the appearances most commonly met with and illustrated are those given in Text-fig. 2.

Attachment of the Crescent to the Red Corpuscle.—The crescent follows in general the same method of attachment to the corpuscle as

do the parasites of the other malarial infections.[2] They encircle with their cytoplasm mounds of hemoglobin substance, which assists them to maintain their rather precarious position on the surface of the corpuscle while they dissolve and digest the hemoglobin. The mounds of hemoglobin to which the corpuscles are attached may be seen protruding through the cytoplasm of the body proper of the crescent as well as at the periphery (Figs. 1 to 105). One might say that the parasite attaches itself to the corpuscle in two ways: (*a*) encircling with its body protoplasm surface mounds of hemoglobin; (*b*) encircling with pseudopodia arising from the cytoplasm peripheral mounds of hemoglobin.

TEXT-FIG. 2, *a*, *b*, and *c*. The crescent wraps itself around the red corpuscle, and proceeds to decolorize it. The so called "bib" is the decolorized corpuscle. When the bib is present it practically always comes from the concavity of the crescentic body as shown in *a*, *b*, and *c*. Ordinarily the bib appears only on one side of the crescent, but occasionally it may be seen on both sides as is schematized in *c*. In these instances the outline of the decolorized corpuscle can usually be traced through the substance of the crescent.

Surface Mounds.—The mounds of hemoglobin substance protruding through the protoplasm of the parasite do not seem to alter the general outline of the crescentic body. In many instances where the apex of the surface mound extends beyond the periphery of the crescentic body, the outline of the crescent may be traced beneath the transparent mound. The mounds protrude through various parts of the body of the crescent, some of them protruding where the nucleoplasm is supposed to be; in fact, in rare cases the chro-

[2] In previous publications I have explained and illustrated the method by which the young parasites of æstivo-autumnal infections (Figs. 1 to 7), as well as the parasites of tertian infections (Figs. 8 to 10), secure their attachment to the external surface of red corpuscles.

matin granules may be seen outlining the hemoglobin mound at its base (Figs. 21 and 79 at oo). Frequently the pigment granules may be seen outlining the base of a hemoglobin mound (Figs. 11, 17, and 44 at x).

Peripheral Mounds.—These are seen along the edges of the crescentic body, and do not appear to have protruded through the body proper of the parasite. I believe that these peripheral mounds are encircled by pseudopodia.

Attaching Pseudopodia.—I do not know whether these pseudopodia are used for the purpose of capturing their prey as well as for the purpose of securing it after it has been captured. They arise from the cytoplasm of the parasite, staining similarly, and may show definitely, especially in specimens where the hemoglobin mounds are seen. As one would expect, considering their purpose, they may be seen either in the form of loops (Figs. 43, 58, 60, 70, 98, and 105 at o), or as strings of cytoplasm (Figs. 21, 34, 56, 57, 59, 79, 97, and 98 at o). The large amount of cytoplasm which may enter into the formation of these pseudopodia is surprising (Figs. 59, 60, 70, 98, 99, and 105).

I have observed these mounds, especially the peripheral mounds, in fresh preparations. This has led me to believe that perhaps some of the small round bodies seen by observers about the periphery of the crescent might be hemoglobin mounds.

Celli and Guarnieri[3] note that "these crescent and ovoid forms may show small round bodies—buds, as it were—about the periphery, one or more in number." Thayer[4] states: "We may observe in certain instances the protrusion of small delicate, bud-like bodies which are cut off from the cell." Celli and Guarnieri suggest that these bodies may represent a method of reproduction, while Thayer states that it is probably a degenerative process. Sforza,[5] judging from certain observations on the staining reactions of crescents, concludes that "the greater part of the crescentic body is nothing more or less than the degenerate red corpuscle." To reach this conclusion, could he have seen the hemoglobin mounds protruding through the crescentic body?

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[3] Celli and Guarnieri, quoted from Thayer, W. S., and Hewetson, J., The malarial fevers of Baltimore, *Johns Hopkins Hosp. Rep.*, 1895, v, 162.

[4] Thayer, W. S., Lectures on the malarial fevers, New York, 1897, 73.

[5] Sforza, quoted from Thayer and Hewetson, The malarial fevers of Baltimore, *Johns Hopkins Hosp. Rep.*, 1895, v, 169.

Decolorization of Corpuscular Mounds by Parasitic Action. Vacuolization.

As a rule, mounds of hemoglobin substance are rather rapidly decolorized (dissolved and assimilated) by the parasite. This may be demonstrated by the examination of a large series of films taken in immediate succession. After decolorization of the surface mounds has taken place, the appearance of the parasite corresponds to what has often been described and pictured as "vacuolization." Mounds in the process of decolorization by the action of the parasite are frequently seen (Figs. 62 to 69). I have observed vacuoles in the living parasite many times and have never considered the process a degenerative one. The vacuoles vary in size and shape and two or more may run together to make one.

The idea that vacuolization is synonymous with degeneration seems to be well fixed in the minds of many observers. Canalis[6] pictures a crescent with vacuoles, describing it as a crescent showing degeneration. Antolisei and Angelini[7] describe "degenerate vacuolating forms which represent the death of the parasite." Mannaberg[8] writes: "In fresh preparations appearances are sometimes seen in the crescents which must be considered to be processes of degeneration. They consist of the appearance of clear circles and spots, which alter their shape under the observer's eye." Marchiafava and Bignami[9] write: "We may also see the process of vacuolization of the crescent bodies as well as of the ovoid and round ones," describing it as a degenerative alteration. Celli and Guarnieri[3] note "vacuolic degeneration of the crescentic forms." Manson[10] writes that the protoplasm of the crescent "shows vacuolation and other signs of degeneration."

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[6] Canalis, P., Studi sulla Infexione malarica Sulla varièta parassitaria delle forme semilunari di Laveran e sulle febbri malariche che da esse dipendono, *Arch. sc. med.*, 1890, xiv, 75, and Plate C, Fig. 11.

[7] Antolisei and Angelini, quoted from Thayer and Hewetson, The malarial fevers of Baltimore, *Johns Hopkins Hosp. Rep.*, 1895, v, 164.

[8] Mannaberg, J., The malarial parasites. A description based upon observations made by the author and other observers; Translation by Felkin, R. W., London, 1894, 287.

[9] Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 47.

[10] Manson, P., Tropical diseases: a manual of the diseases of warm climates, London, Paris, New York, and Melbourne, 2nd edition, 1900, 14.

Thayer[11] states: "Vacuolization of the crescentic, ovoid, and round bodies is not very uncommon. This is usually associated with a diminution of the refractiveness of the parasite and often with a loss of regular outline. The vacuoles are small, but may vary considerably in size, sometimes becoming confluent and larger. The process is evidently degenerative." Thayer and Hewetson[12] state that the "vacuolic degeneration" of crescentic forms is a process which has previously been described by Laveran.

Vacuoles.

Contractile or Pulsatile Vacuoles.—In protozoa the vacuole is not a vacant space. In the fresh water amebæ, in addition to food vacuoles, one may see contractile or pulsatile vacuoles. These vacuoles are exceptional in size and constancy of position. They are usually excretory organs, containing a combination of fluid and gas. At fairly regular intervals they may be seen to contract until they disappear, reforming slowly. But it is not the contractile or pulsatile vacuoles with which we have to do in connection with the malarial parasite, but with the food vacuoles.

Food or Nutritive Vacuoles.—These are usually regarded as of temporary character. They contain liquid, not gas, probably a chemical ferment which dissolves the hemoglobin and makes it available for utilization by the parasite. The parasite assimilates what it needs for nutrition, the waste products being converted into pigment, which is excreted when the parasite segments.

The fresh water ameba resembles the malarial ameba in some respects. It secures its prey by means of pseudopodia which surround the prey, the pseudopodium of the ameba uniting to enclose it within the boundary of its protoplasm, in this way forming a so called nutritive vacuole with the prey as the inclusion. The ameba then proceeds to dissolve the inclusion, absorbing the dissolved material into its substance, storing the reserves, and throwing off the waste products.

I have seen much the same process occur with the mononuclear leukocyte of human blood.[13] The leukocyte put out pseudopodia from

[11] Thayer, Lectures on the malarial fevers, New York, 1897, 72.

[12] Thayer and Hewetson, The malarial fevers of Baltimore, *Johns Hopkins Hosp. Rep.*, 1895, v, 162.

[13] Rowley, M. W., A fatal anæmia with enormous numbers of circulating phagocytes, *J. Exp. Med.*, 1908, x, 78.

its cytoplasm, which captured and included a polynuclear leukocyte, the pseudopodia uniting to form the wall of the nutritive vacuole with the polynuclear leukocyte within it. The mononuclear cell then proceeded to dissolve the inclusion and the gradual disappearance of the structure of the polynuclear leukocyte could be watched.

The vacuoles seen in connection with the malarial parasite are what one would expect to find and are not an indication of degeneration. Grassi[14] shows them in crescentic bodies, Schaudinn[15] pictures vacuoles in the "*ookinete*," and Ruge[16] illustrates them in a proteosoma. Even the decolorized mound ("achromatic area") seen in connection with the young parasite must be a converted nutritive vacuole since the parasite has dissolved and assimilated the hemoglobin which was enclosed within its pseudopodium.

Is it possible that the malarial parasite, like the fresh water ameba, may secrete reserves which would enable it to withstand lack of food for a short time? If this were so, it would suggest what we already know to be necessary—vigorous and long continued treatment.

SUMMARY.

Æstivo-autumnal parasites, including the crescentic bodies, are always extracellular; that is, they are attached to the external surface of the red corpuscles.

Crescentic bodies attach themselves to the red corpuscles just as the younger parasites do, by encircling, with their cytoplasm, mounds of hemoglobin substance. These hemoglobin mounds may be seen protruding through various portions of the crescentic bodies, as well as at the periphery of the parasites. The base of the mounds is occasionally outlined by the chromatin or pigment granules.

The hemoglobin mounds protruding through the body proper of

[14] Grassi, B., Die Malaria, Studien eines Zoologen, Jena, 2nd edition, 1901, Figs. 11, 12, 14 to 17, 20, 21, 24, 25, and 28.

[15] Schaudinn, F., Studien über krankheitserregende Protozoen. II. *Plasmodium vivax* (Grassi & Feletti), der Erreger des Tertianfiebers beim Menschen, *Arb. k. Gsndhtsamte.*, 1903, xix, 169, and Plate 4, Figs. 37, 38, 39, and 40.

[16] Ruge, R., Einführung in das Studium der Malariakrankheiten mit besonderer Berücksichtigung der Technik. Ein Leitfaden für Schiffs- und Colonialärzte, Jena, 1901, Plate 1, Fig. 41.

the crescentic bodies do not seem to alter the general outline of the parasites. The outline of the parasites may be traced through the transparent mounds.

Whenever attaching pseudopodia are observed they are seen to arise from the cytoplasm of the parasites and may be in the form of loops or strings.

When the crescents are attached they proceed to dissolve the hemoglobin to make it available for utilization, assimilating what is required for nutrition, the waste product being in the form of pigment granules.

After the hemoglobin mounds, to which the crescents are attached, have been decolorized by parasitic action, an appearance is obtained which has been described by most observers as vacuolization of the crescentic body. These observers believe the picture to be one of degeneration.

The decolorized mounds or vacuoles ("achromatic areas") seen in connection with malarial parasites correspond to the nutrition vacuoles of the common amebæ, and possibly the malarial parasite may, like these amebæ, secrete reserve food.

EXPLANATION OF PLATES.

PLATE 33.

Magnification, × 1,690.

FIGS. 1 to 7. Young æstivo-autumnal parasites attached to peripheral mounds of hemoglobin substance. The pseudopodia of the parasites have encircled the mounds at their base.

FIGS. 8 to 10. Adult tertian parasites attached to peripheral corpuscular mounds.

FIGS. 11 to 35. Crescentic bodies of æstivo-autumnal infections attached to peripheral and surface mounds of hemoglobin substance. In Figs. 11, 17, 26, and 28 the pigment granules are seen outlining the hemoglobin mounds at their base, at x. In Fig. 21 the chromatin granules may be seen outlining the base of the mound at oo. In Figs. 21 and 34 the pseudopodium may be seen at o.

Figs. 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 33, and 35 correspond to Figs. 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, and 90.

Examination of these pictures with a magnifying glass will show definitely the mounds of hemoglobin substance protruding through the bodies of the crescents.

PLATE 34.

Magnification, × 1,690.

FIGS. 36 to 55. Crescentic bodies attached to peripheral and surface mounds of hemoglobin substance. Fig. 43 shows at o the pseudopodium in the form of a loop. Fig. 44 shows at x pigment granules surrounding the base of the hemoglobin mound. Fig. 45 shows at xx mounds of hemoglobin substance, which was stained a deep pink, reproducing black in the photograph.

Figs. 37, 41, 44, 46, 49, and 55 correspond to Figs. 91, 92, 93, 94, 95, and 96.

FIG. 56. A crescent body attaching to a red corpuscle by means of its pseudopodia, seen at o. This figure corresponds to Fig. 97.

FIG. 57. A crescent attached to a partially decolorized corpuscle. The mounds of hemoglobin substance may be seen and a pseudopodium is seen at o.

FIG. 58. A crescent with an attaching loop of cytoplasm showing at o.

FIG. 59. A crescentic body showing corpuscular mounds and an attaching pseudopodium at o. This figure corresponds to Fig. 98.

FIG. 60. The attaching pseudopodia may be seen at o in the form of loops. This figure shows a large amount of cytoplasm entering into the pseudopodia. This figure corresponds to Fig. 99.

FIG. 61. The corpuscle to which the parasite is attached shows well here, and a careful examination of the parasite will show the surface mounds. This figure corresponds to Fig. 100.

FIG. 62. Here the hemoglobin mounds are showing decolorization. The entire upper end of the parasite is occupied by a partially decolorized mound of hemoglobin protruding through the cytoplasm of the parasite.

FIG. 63. Here one sees the beginning of what is popularly termed vacuolization. The parasite is dissolving and digesting the hemoglobin and the digestive vacuoles can be seen more clearly than when they are filled with the well stained hemoglobin substance. This figure corresponds to Fig. 101.

FIGS. 64 and 65. The dissolving of the hemoglobin in the vacuole, the operation being a little more advanced in Fig. 65. Fig. 64 corresponds to Fig. 102.

FIG. 66. The strands of cytoplasm of the parasite may easily be seen between the vacuoles. The vacuoles are oval in shape and the included hemoglobin almost decolorized. This figure corresponds to Fig. 103.

FIGS. 67 and 68. Definite vacuoles in the body of the parasites. Fig. 68 corresponds to Fig. 104.

FIG. 69. A large vacuole in the parasite.

FIG. 70. The crescentic body here shows that it is attached to corpuscular mounds and a loop arrangement of cytoplasm is seen extending from one end of the parasite to the other end, at o. This figure corresponds to Fig. 105.

PLATE 35.

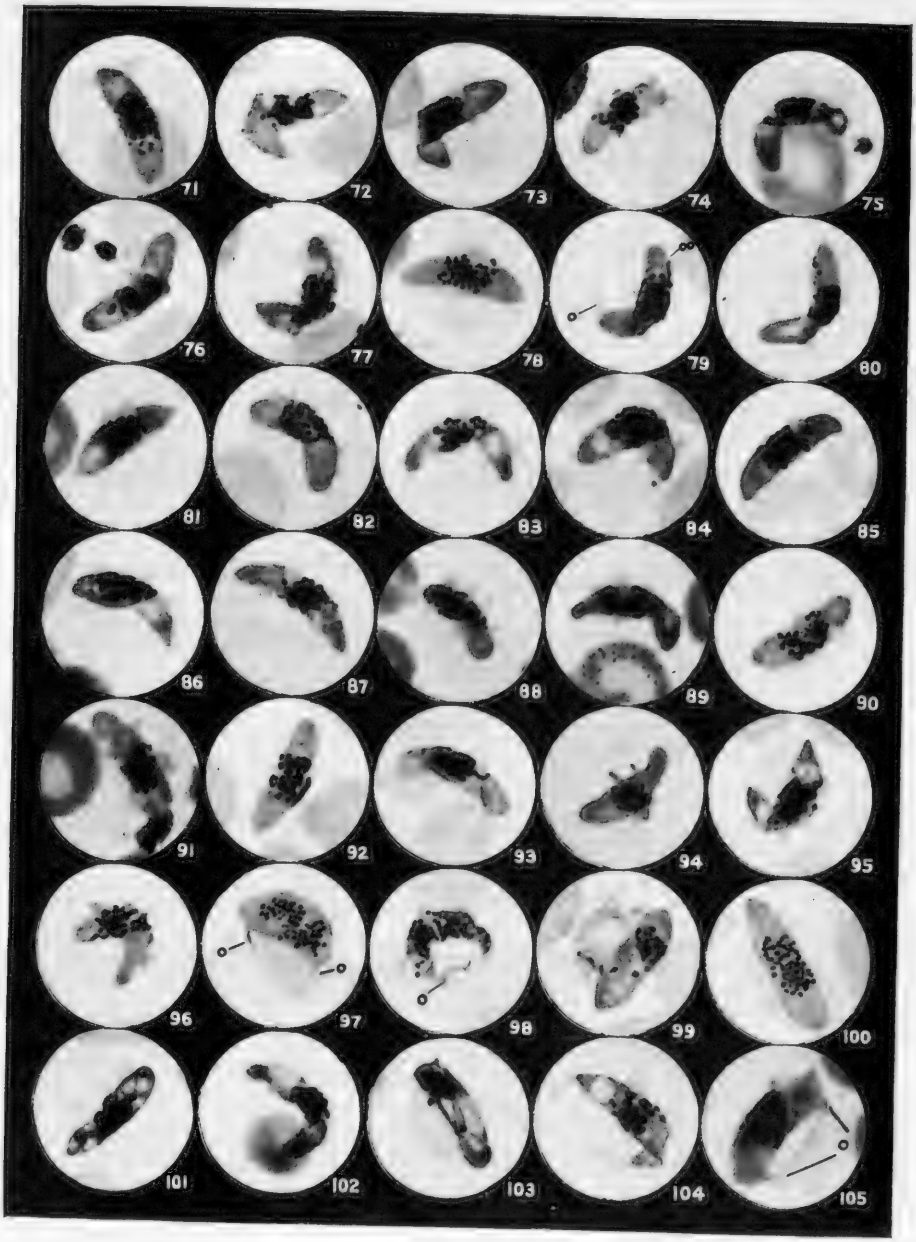
Magnification, × 1,690.

FIGS. 71 to 105. These pictures are colored photographs of certain parasites shown in the black and white reproductions. They show the parasites attached to peripheral and surface mounds of hemoglobin substance. The attaching pseudopodia and the nutritive vacuoles of the parasites can also be seen.

Fig. 71 corresponds to Fig. 12.
Fig. 72 corresponds to Fig. 13.
Fig. 73 corresponds to Fig. 14.
Fig. 74 corresponds to Fig. 15.
Fig 75 corresponds to Fig. 16.
Fig. 76 corresponds to Fig. 18.
Fig. 77 corresponds to Fig. 19.
Fig. 78 corresponds to Fig. 20.
Fig. 79 corresponds to Fig. 21.
Fig. 80 corresponds to Fig. 22.
Fig. 81 corresponds to Fig. 24.
Fig. 82 corresponds to Fig. 25.
Fig. 83 corresponds to Fig. 26.
Fig. 84 corresponds to Fig. 27.
Fig. 85 corresponds to Fig. 28.
Fig. 86 corresponds to Fig. 29.
Fig. 87 corresponds to Fig. 30.
Fig. 88 corresponds to Fig. 31.
Fig. 89 corresponds to Fig. 33.
Fig. 90 corresponds to Fig. 35.
Fig. 91 corresponds to Fig. 37.
Fig. 92 corresponds to Fig. 41.
Fig. 93 corresponds to Fig. 44.
Fig. 94 corresponds to Fig. 46.
Fig. 95 corresponds to Fig. 49.
Fig. 96 corresponds to Fig. 55.
Fig. 97 corresponds to Fig. 56.
Fig. 98 corresponds to Fig. 59.
Fig. 99 corresponds to Fig. 60.
Fig. 100 corresponds to Fig. 61.
Fig. 101 corresponds to Fig. 63.
Fig. 102 corresponds to Fig. 64.
Fig. 103 corresponds to Fig. 66.
Fig. 104 corresponds to Fig. 68.
Fig. 105 corresponds to Fig. 70.

(Lawson: Crescentic bodies in æstivo-autumnal malaria.)

(Lawson: Crescentic bodies in æstivo-autumnal malaria.)



(Lawson: Crescentic bodies in æstivo-autumnal malaria.)

ÆSTIVO-AUTUMNAL PARASITES. MULTIPLE INFECTION OF RED CORPUSCLES AND THE VARIOUS HYPOTHESES CONCERNING IT.*

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Plates 36 to 39.

(Received for publication, February 1, 1918.)

Malarial Parasites.

There are three species of malarial parasites, each with its special morphological and biological characteristics. They are: (1) tertian parasites, (2) quartan parasites, and (3) æstivo-autumnal parasites. It is with the last variety that this paper deals.

Æstivo-Autumnal Parasite.—The young forms of these parasites are very much like those of the tertian and quartan infections, except that, as a rule, the parasites of the æstivo-autumnal infections are relatively smaller than those of the tertian and quartan infections. They are more delicate in appearance, have a more clean-cut outline and a smaller chromatin mass. One should learn to distinguish these morphological differences, as the young parasite may be the only form present at the time of the examination of the blood. The æstivo-autumnal parasites produce the gravest manifestations of the malarial infections so that an early diagnosis may be of the greatest importance.

Multiple Infection of Red Corpuscles with Young Parasites.

Multiple infection of red corpuscles with young parasites is seen in all malarial infections and it is not a rare occurrence in the æstivo-autumnal infections. In films from the circulating blood I have seen from two to seven young parasites on a corpuscle (Figs. 1 to 75).

* Aided by a grant from The Rockefeller Institute for Medical Research.

The occurrence is always accidental and has no significance other than that, if the instances are numerous, it usually means a heavy infection. As a rule, the number of parasites attached to individual corpuscles increases in direct proportion to the severity of the infection; therefore one would not expect to find three, four, or five parasites on a corpuscle in a film where the parasites present were few in number. This fact in itself should suggest that instances of multiple infection have no significance; but various theories have been formulated to explain certain examples.

Theories as to Multiple Infection.—The position of the attached parasites in relation to each other has, apparently, suggested certain theories, such as conjugation ("syngamy," "cytogamy"). The fusion of two cells, cytoplasm to cytoplasm, and chromatin to chromatin, to form a new individual, is a process which has been described frequently in connection with certain protozoa.

Mannaberg[1] was one of the first observers to formulate a theory as to the conjugation of the young amebæ of æstivo-autumnal infections. Two or more parasites were seen attached to adjacent hemoglobin mounds. They were attached so closely together that a portion of the cytoplasm of one parasite was overlying a portion of the cytoplasm of the adjacent parasite. This appearance (Figs. 11 to 30 and 119 to 125) was interpreted by Mannaberg as conjugation. He believed that a fusion of the cytoplasm had taken place, resulting in transitional forms in the formation of the crescents. It would seem that it did not make any difference whether two, three, or four of these young parasites had united to form a crescent, for he states: "I have observed two, or more rarely three, of these parasites may lie closely adhering to one another," and he speaks of "these conglomerate parasites, consisting of two to four specimens." Wright[2] states: "My observations appear to support those of Mannaberg in regard to the genesis of the crescent—the syzygium—from a corpuscle doubly infected by parasites," and "In the stained specimens the syzygies appear to be in the act of conjugation."

As a matter of fact these parasites never unite. The same appearance may be seen in tertian infections.

[1] Mannaberg, J., The malarial parasites. A description based upon observations made by the author and other observers; Translation by Felkin, R. W., London, 1894, 289.

[2] Wright, H., The malarial fevers of British Malaya, *Studies from Institute for Medical Research, Federated Malay States*, Singapore, 1901, i, 4.

Multiple Infection of Corpuscular Mounds.

I have seen from two to five young parasites attached to one corpuscular mound (Figs. 1 to 9, 19, 20 24, 25, 27, 29 to 35, and 37 to 70). Multiple infections of mounds have no significance. The heavier the infection, the more frequently it is seen. The parasites attached to one mound may be in similar or in varying stages of development. The cytoplasm of each parasite is in contact with the hemoglobin mound, and each parasite has a share in its destruction.

Occasionally one finds two parasites attached to one mound so as to give the appearance of a developing crescent (Figs. 31 to 35, 126, and 127), and the same appearance may be seen in tertian infections (Fig. 35). In comparing these instances of æstivo-autumnal and tertian infections of mounds, note the relatively larger size of the chromatin masses of the tertian parasites.

Many theories have been formulated as to the significance of the appearance where two or more parasites encircled one mound of hemoglobin substance, especially by observers who believed the parasites to be within the substance of the corpuscle.

The youngest form of the æstivo-autumnal parasite to attach itself to the red corpuscle may require its entire cytoplasm to encircle a hemoglobin mound. When one of these parasites is so attached, it appears as a ring-form of a delicate, thread-like structure, more or less uniform in size throughout its circumference. If two or more of these tiny parasites encircle the one hemoglobin mound, the cytoplasm of one parasite superimposed over the cytoplasm of the other parasite or parasites, and the chromatin masses separated or lying close together, as accident may direct, the appearance of a single ring with more than one mass of chromatin is obtained (Figs. 1 to 4 and 6).

Marchoux[3] suggested that these forms result from conjugation, and Ewing[4] states that while such an explanation appears reasonable, it is without proof, and the more probable explanation is the incomplete fusion of the chromatin in the rosette.

[3] Marchoux, E., Le paludisme au Sénégal, *Ann. Inst. Pasteur*, 1897, xi, 647.

[4] Ewing, J., Malarial parasitology, *J. Exp. Med.*, 1900–01, v, 482.

As the æstivo-autumnal parasite increases in size, especially if it has developed a thickening of one segment, giving what has been called the "signet-ring" appearance, it is easier to recognize the individual parasites when two or more encircle one mound (Figs. 31 to 34, 36 to 70, 106 to 112, 114 to 118, and 126 to 129).

Craig[5] but follows in the footsteps of the early investigators. He interprets parasites attached to adjoining mounds and parasites attached to one mound as conjugation forms. The theory of conjugation as advanced by him may be summed up in a few words. He states that it occurs "within the infected erythrocytes,"[6] that conjugation "occurs only between two young hyaline forms of the plasmodia, indistinguishable in size and structure,"[7] that it "is completed during the hyaline stage before the formation of pigment,"[8] that the "process occurs in every malarial infection in which quinine has not been given early,"[9] and that it is "the most rational explanation of latency and recurrence in malarial disease."[9] In the same article Craig illustrates[10] two young parasites side by side, a portion of the cytoplasm of one parasite overlying a portion of the cytoplasm of the other parasite. In referring to them he states: "Protoplasmic union is almost complete, and the portions in apposition are beginning to be absorbed." He also pictures[11] two young parasites encircling one corpuscular mound, interpreting the appear-

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[5] Craig, C. F., Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, 285, 318.

[6] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, 300.

[7] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, 304.

[8] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, 309.

[9] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, 301.

[10] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, Fig. 5.

[11] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, Fig. 7.

ance as "a conjugation form in which the two chromatin masses are distinct and the portions of protoplasm in apposition have become absorbed, resulting in the formation of a large ring-like body with two chromatin masses."[12] In his diagrammatic figures Craig advances the stages in his conjugation theory by bringing the chromatin masses closer and closer together until they are side by side. He finally pictures a large ring-form parasite with one mass of chromatin, which he assumes to be "a form resulting after conjugation is completed."[13]

Premature Division of the Chromatin.

In the adult parasite nuclear division seems to go on more rapidly than cytoplasmic, and it may be completed before cell division takes place. But premature division of the chromatin never takes place in the young parasite.

Certain observers have described what they believed to be a precocious division of the chromatin. Ziemann[14] was at first uncertain whether the appearance was due to two fused parasites or to a precocious division of one nucleus, but finally accepted the latter hypothesis, describing "the separation of one, or rarely two, accessory granules from the original mass in cells infected by single parasites. Sometimes the accessory granule was much smaller than, sometimes nearly as large as, the main granule." And Ewing[15] goes on to say: "All of these appearances I have seen in single parasites, less often in single members of conjugating pairs, and I agree with Ziemann as to their significance." Emin[16] gives examples of two and three parasites encircling one corpuscular mound, and interprets the condition as that of precocious division of the chromatin.

Variation in the size of the chromatin masses of young parasites is frequently observed. It may be only an apparent variation in size, or a breaking up of the chromatin due to technique, or it may be a normal occurrence. The nuclei of young parasites may appear

[12] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, 308.

[13] Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuscular conjugation, *J. Infect. Dis.*, 1910, vii, 318.

[14] Ziemann, H., quoted from Ewing, Malarial parasitology, *J. Exp. Med.*, 1900–01, v, 479.

[15] Ewing, Malarial parasitology, *J. Exp. Med.*, 1900–01, v, 479.

[16] Emin, A., Une variété nouvelle du parasite de Laveran, *Bull. Soc. path. exot.*, 1914, vii, 385; Figs. 3 to 5.

to be larger in certain parts of a film, where the red corpuscles are thinly spread, than they do in the thicker portions. This appearance is due to the flattening of the chromatin mass with consequent enlargement. Irregularity in the size and distribution of chromatin masses in the young parasite is frequently due to technique while spreading the film and illustrates how easily the chromatin may be subdivided. Occasionally young parasites in varying stages of development may occupy one corpuscular mound (Figs. 2, 8, 9, 49, 62, 65, 68 to 70, 112, and 114 to 116). In these instances the cytoplasm may vary in amount and the chromatin mass in size. Where several young parasites are attached to a corpuscle the same variation may be seen (Figs. 26, 73 to 75, 117, and 118). Variations in the size of young parasites are to be expected since all adult parasites do not segment at once in any malarial infection.

Multiple Infection of Red Corpuscles with Crescentic Bodies.

This occurrence (Figs. 77 to 105 and 131 to 140), like multiple infection by young parasites, is always accidental. It has no significance other than the fact that, if several instances are seen in one film, a severe infection is indicated. So far as I know, there have been no theories advanced to explain the occurrence of more than one crescent on a corpuscle. I have seen three crescents on a corpuscle (Fig. 100), but rarely. Two is the number usually seen and I believe that two on a corpuscle are considered a rare occurrence.

There is apparently very little literature referring to two crescents on a corpuscle. Marchiafava and Bignami[17] write: "We have also seen two crescents within the same blood corpuscle, the curved portions being face to face." Manson[18] figures two crescents attached to the one corpuscle, and states: "Very rarely twin or double crescents, that is two crescents in one corpuscle—are encountered." Cropper[19] pictures two crescents on a corpuscle.

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[17] Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 42.

[18] Manson, P., Tropical diseases: a manual of the diseases of warm climates, London, Paris, New York, and Melbourne, 2nd edition, 1900, 14.

[19] Cropper, J., Phenomenal abundance of parasites in a fatal case of pernicious malaria, *Lancet*, 1908, ii, 16.

In my experience, one of the rarest occurrences in instances of multiple infection of red corpuscles is the presence of a young parasite and a crescent (Figs. 76 and 130). I have seen but three examples of this.

I am convinced that malarial parasites do not conjugate.

Marchiafava and Bignami[20] state: "Not infrequently several young parasites are seen in the same red corpuscle; we have counted up to six or seven, and when they are very close together they may appear to be intimately adherent. . . . . we cannot hold it to have been conclusively demonstrated that the young parasites collected within one red corpuscle become merged together; on the contrary, they follow their own development."

Many of the morphological and biological phases would cease to be obscure if observers realized that malarial parasites are attached to the external surface of red corpuscles.

SUMMARY.

1. Multiple infection of red corpuscles with young parasites is seen in all malarial infections, but it is found most frequently in the æstivo-autumnal infections. The occurrence is accidental and has no significance other than that if the instances are numerous it suggests a heavy infection.

2. In instances of multiple infection the young parasites may be seen to be attached: (*a*) each encircling its own corpuscular mound, giving the typical ring-form picture, or (*b*) two or more encircling one corpuscular mound, giving the appearance of a single ring with two or more masses of chromatin.

3. Certain hypotheses as to the conjugation of malarial parasites have been formulated by observers to explain various instances of multiple infection. I do not believe that conjugation ever occurs. I believe that these hypotheses resulted from observation of certain appearances presented by the attached parasites, as when they are attached so closely together that they may appear to be adherent, or when two or more are attached to one corpuscular mound, giving

[20] Marchiafava and Bignami, Malaria, in Stedman, Twentieth century practice. New York, 1900, xix, 46.

the appearance of a single parasite with more than one mass of chromatin.

4. Certain appearances have also been described as a precocious division of the chromatin masses of young parasites. In these instances the chromatin granules were usually described as varying in size. Such an appearance may be explained as follows: (*a*) two young parasites in varying stages of development may encircle one corpuscular mound, the cytoplasm of one parasite being superimposed over that of the other parasite, giving a picture of a single ring with two unequal masses of chromatin; or (*b*) the variation in the size and number of the chromatin masses may be the result of traumatism, as the nuclei of young parasites are rather easily broken up.

5. Multiple infection of red corpuscles with crescentic bodies is considered rather a rare occurrence. It is always accidental, and if the instances are numerous it means a severe infection.

6. When one accepts the fact that all malarial parasites are attached to the external surface of the red corpuscles, the biological and morphological characteristics of the parasites cease to be obscure.

EXPLANATION OF PLATES.

PLATE 36.

ÆSTIVO-AUTUMNAL PARASITES (TERTIAN PARASITES, FIG. 35).

Magnification, × 1,840.

FIG. 1. Two very young parasites encircling one surface hemoglobin mound. The cytoplasm of one parasite superimposed over that of the other parasite gives the appearance of a single ring with two chromatin masses. One chromatin mass extends beyond the periphery of the corpuscle.

FIG. 2. Examples of two young parasites encircling one surface hemoglobin mound. The chromatin masses of these parasites vary in size.

FIG. 3. Two young parasites encircling a peripheral hemoglobin mound.

FIG. 4. Three young parasites are attached to this corpuscle; two of them encircle one surface hemoglobin mound at X. Pigment granules are seen at O.

FIG. 5. Two young parasites attached to one surface hemoglobin mound. The nuclei of these parasites are shaped to the mound.

FIG. 6. Three young parasites encircling one surface hemoglobin mound. The parasite at X appears to be more advanced in development than the other two.

FIG. 7. Three young parasites encircling one decolorized hemoglobin mound.

FIG. 8. Four young parasites encircling one surface hemoglobin mound. The chromatin masses vary in size. This figure corresponds to Fig. 112.

FIG. 9. Four young parasites encircling one surface hemoglobin mound. The chromatin masses vary in size.

FIG. 10. Five young parasites are attached to this corpuscle; four of them encircle one surface hemoglobin mound. A pigment granule may be seen at o. This figure corresponds to Fig. 113.

FIGS. 11 to 15. These parasites have developed a thickening of one segment. They encircle, in various positions, adjacent hemoglobin mounds. They are attached so closely together that a portion of the cytoplasm of one parasite is overlying a portion of the cytoplasm of the parasite attached to the adjacent mound (corresponding to Mannaberg's conjugation forms). Figs. 11, 13, and 14 show decolorized hemoglobin mounds in connection with but one of the two parasites, suggesting that one of these parasites attached itself to the red corpuscle before the other parasite did. Pigment granules may be seen at o. Fig. 12 corresponds to Fig. 120.

FIG. 16. Two young parasites encircling one peripheral hemoglobin mound. The apex of the mound is not yet decolorized.

FIGS. 17 to 19. Young parasites are shown encircling adjacent decolorized hemoglobin mounds. In Fig. 17 a pigment granule in connection with one parasite may be seen at o.

FIG. 20. At x two young parasites encircle one hemoglobin mound, and at o the two young parasites are attached to adjacent mounds, a portion of the cytoplasm of one overlying a portion of the cytoplasm of the other parasite.

FIGS. 21 to 23. Young parasites attached to adjacent dehemoglobinized corpuscular mounds. In Fig. 21 pigment granules may be seen at o. Fig. 23 shows two parasites encircling one of the decolorized mounds. The two parasites in Fig. 22 appear to be in a similar stage of development to those seen in Fig. 21, and to have done as much damage to the infected corpuscle, yet pigment granules are seen only in connection with the parasites in Fig. 21. These figures correspond to Figs. 121, 119, and 122.

FIG. 24. Four parasites are attached to this corpuscle. There are two decolorized hemoglobin mounds, each encircled by two parasites.

FIG. 25. Five parasites are attached to the corpuscle, two of them to the peripheral mound at x. Note the variation in size of the chromatin masses.

FIG. 26. Five parasites are attached to the corpuscle. Note the variation in the development of these parasites.

FIG. 27. Six parasites are attached to the corpuscle, two to each hemoglobin mound. Two of the mounds are decolorized; the parasites attached to the third and central mound have attached themselves more recently than the other parasites. This figure corresponds to Fig. 124.

FIG. 28. Six parasites are attached to the corpuscle, two to each hemoglobin mound. These mounds are all decolorized and a pigment granule is seen at o.

FIG. 29. Seven parasites are attached to the corpuscle, two to each decolorized mound and one to the mound which has not yet been decolorized. This figure corresponds to Fig. 125.

FIG. 30. Four parasites are attached to the corpuscle, two to one mound, and two to separate and adjacent mounds. This figure corresponds to Fig. 123.

FIGS. 31 to 35. Examples of two parasites attached to one surface hemoglobin mound. These parasites give the appearance of developing crescents much more than do the parasites attached to adjoining mounds; but they are only accidentally so attached, and not conjugating. Fig. 35 was taken from a tertian infection. Note the relatively larger size of the chromatin masses of the tertian parasites. Three free parasites may be seen in Fig. 34. Figs. 32 and 34 correspond to Figs. 126 and 127.

PLATE 37.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, × 1,840.

FIG. 36. Two young parasites freed from a corpuscle which they have destroyed. This conclusion was reached because of the size of the parasites and the presence of a pigment granule at o.

FIGS. 37 and 38. Examples of young parasites encircling peripheral hemoglobin mounds. The parasites in Fig. 38 are older than those in Fig. 37.

FIG. 39. Two parasites encircling with their pseudopodia a peripheral hemoglobin mound. These parasites are larger than those seen in Figs. 37 and 38. Pigment granules may be seen at o. This figure corresponds to Fig 107.

FIG. 40. Three very young parasites encircling one large peripheral hemoglobin mound. A pigment granule may be seen at o. This is one of the largest peripheral mounds that I have seen. This figure corresponds to Fig 108.

FIG. 41. At o two young parasites encircle a peripheral hemoglobin mound, while at x two parasites, in a later stage of development, encircle one surface hemoglobin mound. This figure corresponds to Fig. 106.

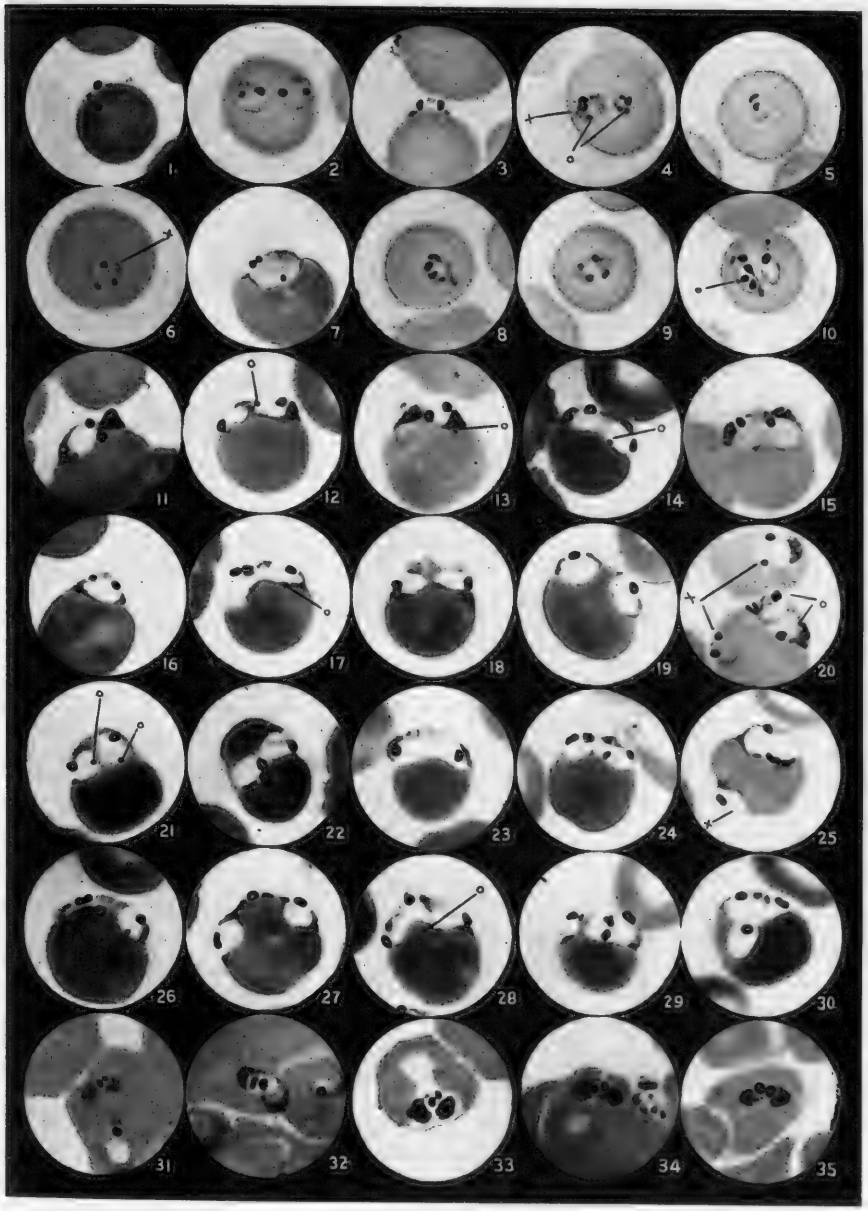
FIGS. 42 to 46. Examples of two parasites attached to one surface hemoglobin mound. These parasites have developed a thickening of one segment. A pigment granule is seen in connection with one of the parasites in Fig. 46. In Figs. 42 and 43 the parasites have destroyed the corpuscle to which they were attached and are free in the position they occupied when attached to the corpuscle.

FIG. 47. There are four parasites attached to this corpuscle; three to one mound, and one to an adjoining mound. Note the small size of one of the chromatin masses. A pigment granule is seen at o.

FIG. 48. Two parasites encircling one surface hemoglobin mound. A pigment granule is seen at o. This figure corresponds to Fig. 109.

FIG. 49. Three parasites attached to one surface hemoglobin mound. Note the variation in the size of the chromatin masses.

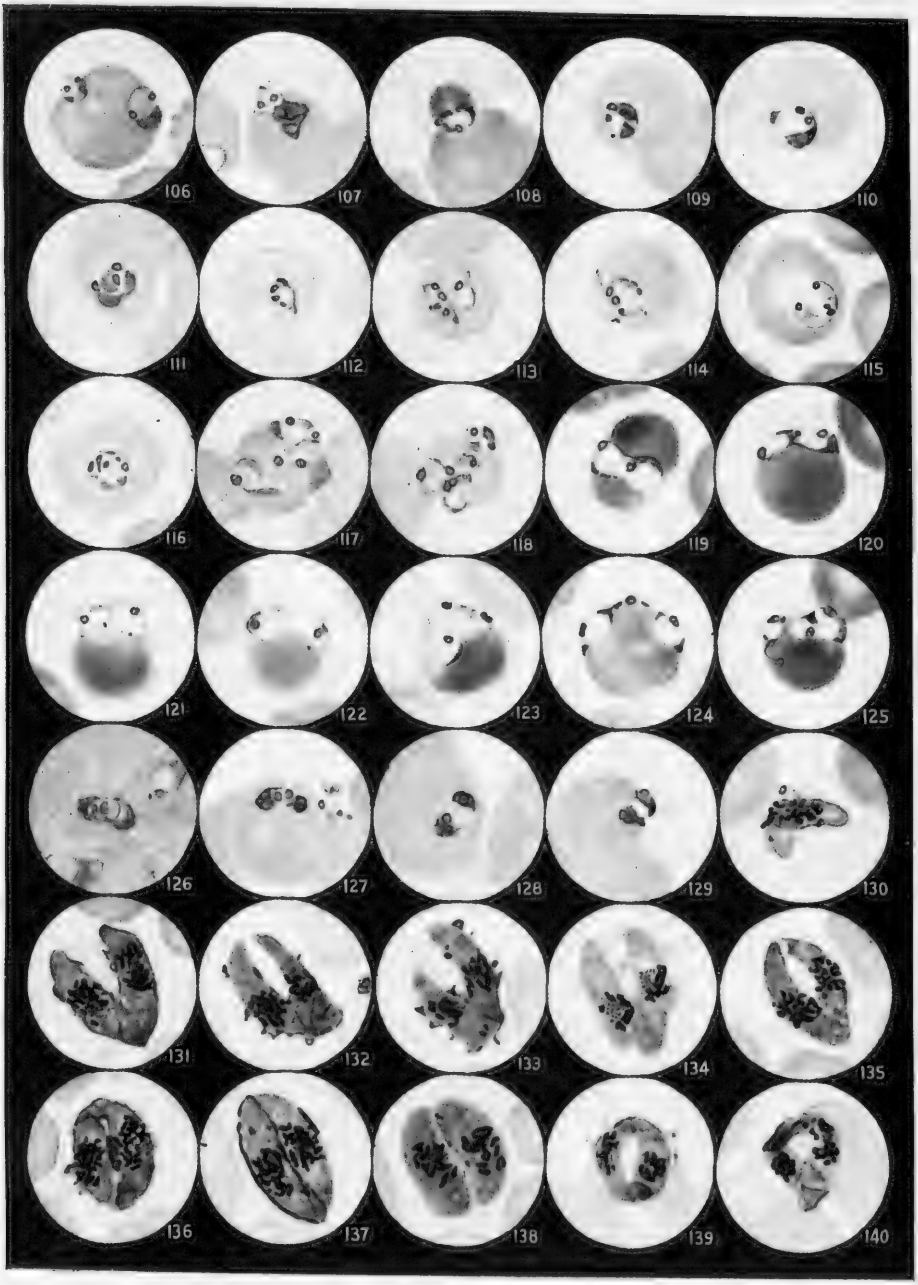
FIG. 50. Two parasites encircling one hemoglobin mound; the body of one of



(Lawson: Multiple infection of red corpuscles.)

(Lawson: Multiple infection of red corpuscles.)

(Lawson: Multiple infection of red corpuscles.)



(Lawson: Multiple infection of red corpuscles.)

the parasites rests on the periphery of the corpuscle. A pigment granule is seen at o.

FIGS. 51 and 52. Examples of young parasites encircling one surface hemoglobin mound. These parasites are in similar stages of development; the hemoglobin mound is larger in Fig. 52 than in Fig. 51.

FIGS. 53 and 54. Examples of two parasites in varying stages of development attached to one surface hemoglobin mound. Fig. 53 corresponds to Fig. 110.

FIGS. 55 to 61. Examples of two parasites encircling, in various positions in relation to each other, one surface hemoglobin mound. Fig. 59 shows four parasites, two attached to each surface hemoglobin mound. Figs. 55 and 56 correspond to Figs. 128 and 129.

FIGS. 62 to 67. Examples of three parasites encircling one hemoglobin mound. Note the variation in the size of the chromatin masses in Figs. 62 and 65. In Fig. 65 the hemoglobin mound to which the parasites are attached is easily seen at the periphery of the corpuscle. Fig. 64 corresponds to Fig. 111.

FIGS. 68 and 69. Examples of four parasites encircling one surface hemoglobin mound. Note the variation in size of the chromatin masses. These figures correspond to Figs. 114 and 115.

FIG. 70. Five young parasites encircling one surface hemoglobin mound. There is quite a variation in the size of the chromatin masses. This figure corresponds to Fig. 116.

PLATE 38.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, × 1,840.

FIGS. 71 to 75. Instances of multiple infection of red corpuscles by young parasites (four to seven). Each chromatin mass corresponds to one or two parasites. In Fig. 71 the parasites are very young and have attached themselves to the periphery of the corpuscle, two at the top, two at the right, and two at the left. Fig. 72 shows four parasites, Fig. 73, seven parasites, and Figs. 74 and 75, five parasites. In Figs. 72 to 75 note the variation in size of the chromatin masses. Figs. 73 and 74 correspond to Figs. 118 and 117.

FIG. 76. A crescent and a young parasite attached to the same red corpuscle. This figure corresponds to Fig. 130.

FIGS. 77 to 98 and 101 to 105. Examples of two crescents attached to one red corpuscle. In Figs. 83, 86 to 88, 90 to 93, 96, and 101, the mounds of hemoglobin substance to which the crescents have attached themselves are well shown. In Fig. 93 the hemoglobin mounds have been dehemoglobinized by the parasites. In Fig. 94 a pseudopodium arising from the cytoplasm of the parasite is shown in the form of a large loop.

Figs. 81, 82, 83, 84, 86, 87, 88, 90, 101, and 105 correspond to Figs. 135, 137, 136, 138, 133, 132, 131, 134, 139, and 140.

FIG. 99. This figure may be variously interpreted. It may be two contracted crescents, it may be a crescent twisted on itself, or it may be a segmenting crescent.

FIG. 100. Three crescents attached to one corpuscle. This showed better in the stained specimen than it does in the photograph.

PLATE 39.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, × 1,840.

FIG. 106. Four young æstivo-autumnal parasites attached to a corpuscle; two encircle a peripheral hemoglobin mound, and two, in a more advanced stage of development, encircle a surface hemoglobin mound. This figure corresponds to Fig. 41.

FIG. 107. Two young æstivo-autumnal parasites, with abundant cytoplasm, encircling a peripheral hemoglobin mound. Two pigment granules are seen in connection with one of the parasites. This figure corresponds to Fig. 39.

FIG. 108. Three very small æstivo-autumnal parasites encircling a very large peripheral hemoglobin mound. This figure corresponds to Fig. 40.

FIG. 109. Two young æstivo-autumnal parasites, in similar stages of development, encircling one surface hemoglobin mound. A pigment granule is seen in connection with one of the parasites. It is easily seen that these are individual parasites. This figure corresponds to Fig. 48.

FIG. 110. Two young æstivo-autumnal parasites, in varying stages of development, encircling one surface hemoglobin mound. This figure corresponds to Fig. 53.

FIG. 111. Three young æstivo-autumnal parasites, in similar stages of development, encircling one surface hemoglobin mound. This figure corresponds to Fig. 64.

FIG. 112. Four very young æstivo-autumnal parasites encircling one surface hemoglobin mound. Note the variation in size of the chromatin masses. This figure corresponds to Fig. 8.

FIG. 113. Five parasites are attached to this corpuscle. Four of them encircle one surface hemoglobin mound; a pigment granule is seen in connection with one of these. This figure corresponds to Fig. 10.

FIG. 114. Four young æstivo-autumnal parasites encircling one surface hemoglobin mound. These parasites are in varying stages of development. Note the variation in size of the chromatin masses. This figure corresponds to Fig. 68.

FIG. 115. Four young æstivo-autumnal parasites, in varying stages of development, encircling one surface hemoglobin mound. One of the chromatin masses is slightly smaller than the others. This figure corresponds to Fig. 69.

FIG. 116. Five young æstivo-autumnal parasites, in varying stages of development, attached to one surface hemoglobin mound. Note that one of the chromatin masses is distorted. This figure corresponds to Fig. 70.

FIG. 117. Five young æstivo-autumnal parasites attached to surface hemoglobin mounds. Note that these parasites are not all in similar stages of development. This figure corresponds to Fig. 74.

FIG. 118. Seven young æstivo-autumnal parasites attached to surface hemoglobin mounds. Note the small size of one of these parasites. This figure corresponds to Fig. 73.

FIG. 119. Two young æstivo-autumnal parasites attached to adjacent decolorized hemoglobin mounds. This figure corresponds to Fig. 22.

FIG. 120. Two young æstivo-autumnal parasites encircling two decolorized hemoglobin mounds. A granule of pigment may be seen in connection with one of these parasites. A portion of the cytoplasm of one parasite is seen to overlie a portion of the cytoplasm of the parasite attached to the adjacent hemoglobin mound. This figure corresponds to Fig. 12.

FIG. 121. Two young æstivo-autumnal parasites attached to adjoining hemoglobin mounds. The mounds are decolorized and a pigment granule is seen in connection with each parasite. This figure corresponds to Fig. 21.

FIG. 122. Three young æstivo-autumnal parasites are attached to this corpuscle. Two encircle the decolorized hemoglobin mound at the right (a pigment granule is seen in connection with them), and one encircles the decolorized mound at the left. This figure corresponds to Fig. 23.

FIG. 123. Four young æstivo-autumnal parasites are attached to this corpuscle. Two of them encircle one decolorized hemoglobin mound. This figure corresponds to Fig. 30.

FIG. 124. Six young æstivo-autumnal parasites are attached to this corpuscle, two attached to each hemoglobin mound. Two of the hemoglobin mounds have been decolorized by the action of the parasites. This figure corresponds to Fig. 27.

FIG. 125. Seven young æstivo-autumnal parasites are attached to this corpuscle. Two parasites encircle each of the three decolorized hemoglobin mounds; pigment granules are seen in connection with these parasites. This figure corresponds to Fig. 29.

FIG. 126. Two young æstivo-autumnal parasites attached to one surface hemoglobin mound. This figure corresponds to Fig. 32.

FIG. 127. Two young æstivo-autumnal parasites attached to one surface hemoglobin mound. To the right may be seen three young parasites free. This figure corresponds to Fig. 34.

FIGS. 128 and 129. Examples of two young æstivo-autumnal parasites attached to one surface hemoglobin mound. One of the two parasites in Fig. 129 rests on the periphery of the corpuscle. These figures correspond to Figs. 55 and 56.

FIG. 130. A young æstivo-autumnal parasite and a crescent attached to the same red corpuscle. This figure corresponds to Fig. 76.

FIGS. 131 to 140. Examples of two crescents on one corpuscle. In Figs. 131 to 136 and 139, the mounds of hemoglobin substance to which the crescents are attached are well shown. These figures correspond to Figs. 88, 87, 86, 90, 81, 83, 82, 84, 101, and 105.

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